University of Groningen

Characterization of genes involved in the regulation of programmed cell death in Arabidopsis thaliana

Qureshi, Muhammad Kamran

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2012

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Qureshi, M. K. (2012). *Characterization of genes involved in the regulation of programmed cell death in Arabidopsis thaliana*. s.n.

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

Download date: 28-12-2022

University of Groningen

Characterization of genes involved in the regulation of programmed cell death in *Arabidopsis thaliana*

Muhammad Kamran Qureshi

The studies described in this thesis were carried out in the Department Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen. The research was financially supported by the Higher Education Comission (HEC), Pakistan and the University of Groningen, The Netherlands.

Printed: CPI, Wöhrmann print service

Cover design: M.K. Qureshi

ISBN: 978-90-367-5491-0

RIJKSUNIVERSITEIT GRONINGEN

Characterization of genes involved in the regulation of programmed cell death in *Arabidopsis thaliana*

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 4 mei 2012
om 12.45 uur

door

Muhammad Kamran Qureshi
geboren op 27 september 1981
te Multan, Pakistan

Promotor:	Prof. dr. J. Hille
Copromotor:	Dr. T.S. Gechev
Beoordelingscommissie:	Prof. dr. J.T.M. Elzenga Prof. dr. J.D. van Elsas Prof. dr. J. Kok

Dedicated to my parents Mr. & Mrs. Riaz Ahmad Qureshi

Contents

	Scope of the thesis	9
Chapter 1	Reactive oxygen species and control of cell death in higher plants	15
Chapter 2	*Arabidopsis* AAL-toxin-resistant mutant *atr1* shows enhanced tolerance to programmed cell death induced by reactive oxygen species	59
Chapter 3	Isolation and characterization of *Arabidopsis* mutants with enhanced tolerance to oxidative stress	77
Chapter 4	Isolation and characterization of genes from *atr2* and *atr7* that confer enhanced tolerance to oxidative stress in *Arabidopsis thaliana*	97
Chapter 5	The zinc finger protein ZAT11 modulates paraquat-induced programmed cell death in *Arabidopsis thaliana*	117
	Summary	141
	Samenvatting	147
	Acknowledgements	153

Scope of the thesis

Scope of the thesis

Necrotrophic fungal pathogens infect plant tissues by secreting specific phytotoxins into the living cells and triggering cell death. Among these phytotoxins, the fungal *Alternaria alternata* f. sp. *lycopersici*-toxins (AAL-toxins) are well characterized inducers of programmed cell death (PCD) in tomato. AAL-toxins inhibit the activity of ceramide synthase and disrupt sphingolipid metabolism. This results in depletion of complex ceramides and accumulation of free sphingoid bases. Tolerance to AAL-toxins in tomato is governed by a single gene, *Asc* (*Alternaria* stem canker).

AAL-toxins treatment, in addition to disruption of sphingolipid metabolism, leads to accumulation of reactive oxygen species (ROS), an event preceeding PCD. ROS, including singlet oxygen, superoxide radical, hydroxyl radical, and hydrogen peroxide, are not only toxic by-products of cellular metabolism in all eukaryotic organisms but also signaling molecules that regulate processes such as plant growth, development and cell death. ROS can induce defense mechanisms or PCD in a dose dependent manner. The production of high amounts of ROS stimulated by external agents can trigger PCD in the plant cells.

PCD is also an essential part of plant life. In addition to growth and development, PCD may be required for proper responses to the environment. It is the systematic removal of undesirable or severely damaged cells, triggered by internal or external stimuli (e.g. pathogen attack, ultraviolet-light, ozone, 3-aminotriazole (AT) and methyl-viologen/paraquat (PQ)). The **aim of this thesis** research is to better understand the mechanisms underlying PCD and to identify potential regulators involved in cell death in the model plant species *Arabidopsis thaliana*. Both AAL-toxins and ROS inducing agents such as AT and PQ are exploited in order to study molecular and genetic aspects of PCD.

The PCD process in animals is well characterized, whereas relatively little is known about PCD in plants. The introductory **chapter 1** gives an overview of the current knowledge about the role of PCD during different developmental processes and during stress responses. Possible roles of AAL-toxins and ROS in PCD are

discussed. The contribution of various downstream candidate genes in the PCD process is part of the chapter.

In order to study AAL-toxins induced PCD in more detail, a T-DNA mutant in *Arabidopsis thaliana* was identified. This knockout, called *loh2* (LAG one homologue2) and is homologous to the *Asc* gene in tomato, rendered mutant plants sensitive to growth inhibition by AAL-toxins. For in depth analysis of AAL-toxins induced PCD in *Arabidopsis*, genetic screening of the progeny of chemically mutagenized *loh2* seeds in the presence of AAL-toxins was carried out. This led to the identification of nine *atr* (AAL-toxin resistant) mutants. In **chapter 2**, the *loh2* genotype and one of the *atr* mutants (*atr1*) are characterized with respect to genetic behaviour and towards the ROS-induced PCD. Questions regarding molecular interactions between ROS and redox signaling are addressed by comparing the transcriptomes regulated in *loh2* and in *atr1*. Is there a molecular link between sphingolipid metabolism and redox signaling during the PCD process? In addition, questions are addressed like are there differences in transcriptional reprogramming during the onset of cell death in ROS sensitive (*loh2*) and tolerant (*atr1*) mutants.

In **chapter 3**, additional *atr* mutants along with *loh2* are characterized phenotypically. The life cycle of *atr* mutants is studied in order to identify similarities/differences at various developmental stages. The tolerance towards ROS-induced cell death of some *atr* mutants is evaluated. Questions are addressed regarding the potential link between oxidative stress tolerance, plant growth and development.

The isolation of gene(s) responsible for the *atr* mutant phenotypes is carried out with the aim to comprehend the molecular nature of these mutants. A map-based cloning approach is an efficient method to identify point mutations. **Chapter 4** describes the characterization of two *atr* mutants through map-based cloning, in combination with massive parallel sequencing. Questions like, what are the characteristic features of the identified genes and the role of the respective mutations, are part of this chapter.

Microarray analysis identified a huge array of genes regulated during AAL-toxins or AT induced cell death in the *loh2* genotype. Some of the regulated genes

were not previously linked to PCD. In addition, functional characteristics of some of the genes were not elucidated. In **chapter 5**, it is attempted to identify regulators at the molecular and the genetic level with a potential role in AAL-toxins- and ROS-induced cell death. Here, questions are addressed like is there a role of early regulated gene(s), altered during AAL-toxins- and ROS-induced stress, during the PCD process. Are the regulated genes involved in the initiation of PCD and/or do those have a role in the ROS signaling pathway? The identification and characterization of such regulators will enhance our understanding of the PCD signaling cascade.

Chapter 1

Reactive oxygen species and control of cell death in higher plants

Muhammad Kamran Qureshi[1]
Neerakkal Sujeeth[1,2]
Veselin Petrov[2]
Tsanko S. Gechev[2]
Jacques Hille[1]

[1] Department Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands

[2] Department of Plant Physiology and Plant Molecular Biology, University of Plovdiv, 24 Tsar Assen str., Plovdiv 4000, Bulgaria

Abstract

Programmed cell death (PCD) is an integral part of the life cycle of eukaryotes and is a genetically controlled process. It is essential for growth, development, and maintaining the tissue and organ homeostasis in plants. PCD also takes place as a response to various environmental stress stimuli. Reactive oxygen species (ROS) play an important role in inducing the PCD process. ROS are toxic by-products of cellular metabolism and their levels are monitored by elaborate detoxification mechanisms. Prolonged stress conditions lead to accumulation of ROS that can trigger a PCD pathway. Disruption of sphingolipid metabolism by external stimuli can also lead to PCD. For example, the fungal AAL-toxins (*Alternaria alternata* f. sp. *lycopersici*) induce cell death by inhibiting ceramide synthase activity, a key enzyme in sphingolipid metabolism. The link between ROS, sphingolipid homeostasis and PCD has been characterized only to a limited extent in plants. In this review, the emphasis will be on the involvement of ROS and sphingolipid metabolism in the induction and execution of the PCD cascade in plants. Additionally, different contributors involved in the PCD signaling machinery in plants will be discussed.

Key words: Programmed cell death; reactive oxygen species; *Alternaria alternata* f. sp. *lycopersici*; sphingolipid

1. Introduction

While animals can move from place to place in search of food and to avoid unfavourable conditions, plants are mainly static and therefore encounter many stress conditions such as drought, salinity, extreme temperature, pollutants and pathogen infection during their life cycle. Plants have evolved ways to use nutrients in the best possible way as well as different mechanisms to cope with stresses (biotic and abiotic). In response to these stresses, an increased production of reactive oxygen species (ROS) takes place, which ultimately can lead to programmed cell death (PCD). Plants also use PCD as a defense strategy against such odds.

ROS is a collective term used for superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide. These components are obligate byproducts of aerobic metabolism in plants. ROS production takes place in the chloroplasts, the mitochondria, the peroxisomes, in the cytosol, at the plasma membrane and in the apoplastic spaces. If remained unchecked, ROS can cause damage to proteins, pigments, lipids and DNA. Therefore, plants have developed mechanisms to detoxify ROS. There are elaborate detoxification/antioxidant systems present in chloroplasts, mitochondria and peroxisomes (as reviewed by Apel and Hirt, 2004; Gechev et al., 2006; Halliwell, 2006; Mittler et al., 2004). However, prolonged stress conditions can overwhelm the scavenging potential of the plant antioxidant systems, which ultimately results in the death of the affected plant cells (Shinozaki et al., 2003; Shinozaki and Yamaguchi-Shinozaki, 2007). The chemical properties of ROS define the specificity and selectivity of ROS signals during stress conditions and during PCD. In addition, the site of production, intensity, developmental stage, plant history and interaction with other signaling molecules are crucial contributing factors in ROS signaling. PCD can be initiated by all kinds of ROS, but the most studied ROS signal is H_2O_2. It can trigger protective mechanisms or PCD in a dose dependent manner (as reviewed by Gechev and Hille, 2005)

PCD is a complex genetically controlled process triggered by intracellular signals, including ROS. It constitutes the elimination of unwanted or severely damaged cells in eukaryotes. The rationale of this ordered destruction of cellular units is the conservation of nutrients, which can be used by other parts of the plant.

PCD in higher plants occurs naturally throughout their life cycle. It is required during plant development processes such as embryo development, maturation of tracheal elements and epidermal trichomes, formation of lace leaf shape, fertilization and leaf senescence (Gechev et al., 2006; Gunawardena et al., 2004; Ito and Fukuda, 2002; Obara et al., 2001; Palma and Kermode, 2003; Van Breusegem and Dat, 2006). PCD can also be observed during the hypersensitive response (HR). This is a defense reaction in which plant cells in and around the site of a pathogen infection die, in order to restrict the spread of the pathogen. Some fungal necrotrophic pathogens can secrete toxins that cause death of healthy cells and tissues so that the pathogens can feed on the dead cells (Gechev et al., 2004). The fungal toxins trigger a response at the site of infection that results in an oxidative burst and subsequently leads to PCD.

Here, we review different phenomena that cause PCD in higher plants. We highlight mainly the parts of the ROS network, including signaling molecules and transcription factors (TF). Moreover, the role of AAL-toxins (*Alternaria alternata* f. sp. *lycopersici*) and sphingolipids will be discussed in relation to the processes that result in PCD.

2. ROS homeostasis

Aerobic organisms like plants use diatomic oxygen as the ultimate electron acceptor. Although atmospheric oxygen is relatively non-reactive, it can give rise to highly reactive intermediates during reduction reactions in a number of metabolic processes such as photosynthesis and respiration. Such intermediates include singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$) (figure 1). These highly reactive compounds have the ability to damage a variety of biological molecules, including DNA and are considered undesirable by-products of cellular metabolism.

Singlet oxygen ($^1O_2$) is synthesized during photosynthesis in the presence of excessive light or low CO_2 concentration. Photosynthesis in high light conditions may lead to the formation of a chlorophyll triplet state. The energy from a chlorophyll triplet state is transferred to oxygen, which ultimately results in $^1O_2$

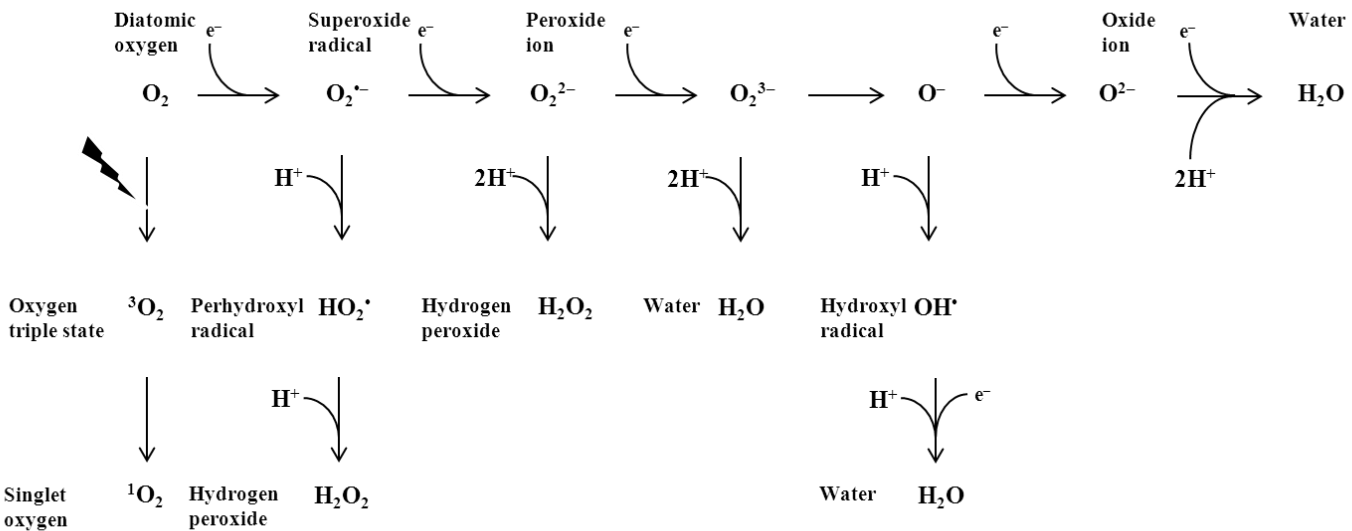
production from the ground triplet $^3O_2$ state. Singlet oxygen has a relatively short half-life of about 3 μs in the cell. If not reduced by carotenoids, tocopherol or plastoquinone, it can lead to the peroxidation of poly-unsaturated fatty acids. Carotenoids together with tocopherols either quench $^1O_2$ directly or prevent its formation by quenching triplet chlorophyll (Krieger-Liszkay et al., 2008).

The partial reduction of oxygen by the transfer of electrons from photosystem I or from reduced ferredoxin to O_2 results in the formation of O_2^- from hydroperoxide radicals ($HO_2^\bullet$). This leads further to the production of hydrogen peroxide (H_2O_2) as *in vivo* O_2^- is quickly dismutated to H_2O_2 by the enzyme superoxide dismutase (SOD). H_2O_2 is a highly reactive, toxic and relatively stable molecule. Therefore, H_2O_2 has the ability to migrate from the site of its production to other cellular locations and to adjacent cells. H_2O_2 has a mild oxidizing power but it is sufficient to inactivate enzymes by oxidizing their thiol group. O_2^- can inactivate important metabolic enzymes containing Fe-S clusters and alters their catalytic activities (Gechev et al., 2006).

The hydroxyl radicals ($HO^\bullet$), are produced from O_2^- or H_2O_2 by using metals as a catalyst in the so called Fenton reaction, previously known as the Haber-Weiss reaction (Koppenol, 2001). It has been reported that $HO^\bullet$ can be formed in the thylakoid membranes of chloroplasts by free metals and reduced ferredoxin (Snyrychova et al., 2006). The hydroxyl radical is the most destructive among all the ROS and destroys virtually anything to which it is exposed. This includes damage to DNA, proteins, lipids and enzymes. Plants do not have mechanisms to detoxify the highly reactive $HO^\bullet$. Therefore, they eliminate its potential producers, like O_2^- and H_2O_2 (Gechev et al., 2006).

Plants are always at risk of oxidative damage as ROS are produced in different cellular compartments. The major sites of ROS accumulation are the chloroplasts. As already mentioned, during photosynthesis, oxygen can accept single electrons passing through the photosystems, which results in the formation of ROS. Abiotic stresses such as excess light, low CO_2 concentrations, drought and salt stresses enhance the ROS concentration in chloroplasts. Plant mitochondria are another site of production of ROS, such as O_2^- and H_2O_2. The ROS sources in

Figure 1: Chemistry of reactive oxygen species (ROS). Production of ROS in the plant cell by the reduction of molecular oxygen under different environmental situations.



mitochondria are the ETC complex III, NADH dehydrogenase and the ubiquinone radical. In normal physiological conditions, mitochondrial O_2^- is rapidly reduced to H_2O_2 by SOD. Mitochondrial ROS play an important role in the regulation of different cellular processes, including PCD. Peroxisomes are spherical microbodies with an oxidative type of metabolism. The main source for ROS generation in peroxisomes is the photorespiration cycle. Some of the other sites of ROS generation within plant cells are the glyoxysomes and the plasmalemma where ROS are produced by fatty acid oxidation and NAD(P)H oxidases respectively (Gechev et al., 2006).

Plants possess intricate mechanisms to detoxify ROS. There are two types of antioxidants, viz. enzymatic and non-enzymatic. Enzymatic antioxidants include catalases (CAT), superoxide dismutases (SOD), ascorbate peroxidases (APX), peroxiredoxins (Prx), glutathione reductases (GR), guaiacol peroxidases (POX), glutathione peroxidases (GPX), glutathione-S-transferases (GST), thioredoxins (Trx), glutaredoxins (Grx), ferritins and alternative oxidases (AOX). Non-enzymatic antioxidants include ascorbic acid, α-tocopherol, carotenoids and flavonoids (table 1).

CATs are the principal scavenging enzymes present in all organisms exposed to internal ROS production. CATs are tetrameric heme containing enzymes, which decompose H_2O_2 into water and oxygen, in an energy-efficient manner. In *Arabidopsis*, there are different CAT isozymes (CAT1, CAT2 and CAT3), encoded by a small gene family. CAT1 is a seed specific catalase, also activated during later stages of plant growth. CAT2 action is mainly confined to photosynthetic tissues, whereas CAT3 is operational throughout the plant growth. CAT2 expression is down-regulated during bolting at the onset of leaf senescence, as H_2O_2 acts as an important signaling molecule during senescence (Zimmermann et al., 2006). The G-Box Binding Factor1 (GBF1), a DNA-binding protein of the CAT2 gene promoter, acts as a negative upstream regulator of CAT2 expression. Overexpression of GBF1 results in a decrease of CAT2 activity and an increase of H_2O_2 levels. Accordingly, CAT2 activity in *gbf1* mutants does not decrease during bolting, H_2O_2 levels remain lower, and as a consequence senescence is delayed (Smykowski et al., 2010).

Catalases, especially CAT2, are involved in the detoxification of peroxisomal H_2O_2 in a day length dependent manner. It was reported that H_2O_2 is unable to provoke cell death symptoms in an *Arabidopsis cat2* mutant grown in short day period (SD). On the other hand, mutant plants grown during the long day period (LD) developed death symptoms triggered by H_2O_2. The pathogenesis related genes (PRs) and camalexin, a phytoalexin, were also induced in *cat2* mutants only during the LD.

SODs are ubiquitous in different sub-cellular compartments of all aerobic organisms. SODs catalyze the dismutation of O_2^- into H_2O_2 and O_2. They are classified based on their metal co-factors (Cu/Zn, Mn, Fe-SOD). All forms of SODs are encoded by nuclear genes and are targeted by amino terminal targeting sequences to the respective organelles (Mittler et al., 2004).

Another enzyme involved in the scavenging of H_2O_2, is ascorbate peroxidase (APX). The enzyme detoxifies H_2O_2 in the presence of ascorbate as a substrate. Studies of mutants revealed that APX is important for protection of the chloroplasts against ROS toxicity induced by light stress (Davletova et al., 2005a; Kangasjarvi et al., 2008; Pnueli et al., 2003).

Although ROS are toxic to cells and quickly quenched by antioxidants, they also have the ability to interact with other signaling molecules, like nitric oxide ($NO^•$), plant hormones like ethylene, certain lipid messengers and are also involved in plant growth. This makes them very versatile molecules. $NO^•$ is capable of interacting with both H_2O_2 and O_2^- in a series of complex reactions triggering cell death and activating the HR response (Delledonne et al., 2001). $NO^•$ production can also be induced by either exogenous application of H_2O_2 or its endogenous generation through ABA (Neill et al., 2008). The interaction pattern of plant hormones with ROS determines the specificity of the ROS signaling pathway (Gechev et al., 2006). There is a strong interplay between ROS and ABA during signal perception, especially in stomatal guard cells, where ABA and H_2O_2 are thought to be involved in the regulation of stomatal closure during periods of salinity, drought and extreme temperatures (Bright et al., 2006; Hirayama and Shinozaki, 2007). ROS regulated by peroxidases are important factors for cell proliferation and differentiation. The ratio between O_2^- and H_2O_2

Table 1: Overview of ROS scavenging enzymes and antioxidants in plants.

Antioxidants	Localized in the cell	Function	Genes involved	Reference
Enzymatic				
Catalases (CAT)	mit, per, gly	Decompose H_2O_2 into water and oxygen. Consist of different isozymes.	Encoded by three genes in *Arabidopsis*. CAT1 is seed specific, CAT2 is active in green tissues, and CAT3 is active in the entire plant.	Zimmermann et al., 2006
Superoxide dismutases (SOD)	cyt, chl, mit, per	Dismutate O_2^- into H_2O_2. Reconized by their metal co-factors	Encoded by eight genes in *Arabidopsis*.	Mittler et al., 2004
Ascorbate peroxidases (APX)	cyt, chl, mit, per	Detoxify H_2O_2 in the presence of ascorbate as a substrate	Encoded by nine genes in *Arabidopsis*.	Gechev et al., 2006
Alternative oxidases (AOX)	mit, chl	Reduce the chance of O_2^- formation during the process of electron transport chain in mitochondria, involves in carotenoid biosynthesis	*Arabidopsis* genome encodes five AOX genes: *AOX1a, AOX1b, AOX1c, AOX1d* and *AOX2*.	Saisho et al., 1997; Thirkettle-Watts et al., 2003
Peroxiredoxins (Prx)	nuc, chl, mit, cyt	Thiol dependent peroxidases, detoxify H_2O_2	Gene family comprises ten family members in *Arabidopsis*	Dietz, 2003

Glutathione peroxidases (GPX)	mit, chl, cyt, er	Detoxification of H_2O_2 and lipid hydroperoxides together with glutathione oxidation	*AtGPX1-AtGPX8*	Rouhier and Jacquot, 2005
Glutathione reductases (GR)	mit, chl, per, cyt	Catalyse the reduction of oxidized glutathione in the presence of NADPH	AtGR1 and *AtGR2*	Gechev et al., 2006; Mhamdi et al., 2010
Guaiacol peroxidases (POX)	mit, vac, cyt, cw	Detoxify H_2O_2 but also involved in the production of ROS, also plays role in variety of plant functions	Seventy three members of the gene family in *Arabidopsis*	Gechev et al., 2006
Monodehydroascorbate reductases (MDHAR)	mit, chl, cyt	Reduce MDHAR radicals in the presence of NAD(P)H	Five members of the gene family in *Arabidopsis*	Mittler et al., 2004
Dehydroascorbate reductases (DHAR)	mit, cyt, chl	Reduce dehydroascorbate	Five members of the gene family in *Arabidopsis*	Mittler et al., 2004
Non-Enzymatic				
Glutathione	mit, chl, cyt, per, vac, apo	Detoxify H_2O_2, $HO_2^{\bullet}$ and other toxic compounds. Acts as substrate for GPX, GR and peroxidases		Dat et al., 2000
Flavonoids	vac	Involved in H_2O_2 and $HO^{\bullet}$ scavenging		Gechev et al., 2006
α-tocopherol	mem	Involved in $^1O_2$ quenching, also involves in lipid peroxides		DellaPenna and Pogson, 2006
Carotenoids	chl, chp, elp, amp	Involved in $^1O_2$ quenching		DellaPenna and Pogson, 2006

The abbreviations are: mit, mitochondria; cyt, cytoplasm; chl, chloroplasts; per, peroxisomes; gly, glyoxysomes.

concentration is the crucial determinant in deciding the cellular proliferation to differentiation rate in the root meristem. The accumulation of O_2^- results in cellular proliferation, whereas the increase in H_2O_2 leads to cellular differentiation. Therefore, when the proportion of O_2^- to H_2O_2 reaches a certain level, the cell proliferation process halts and the cell elongation process begins. The *Arabidopsis* UPBEAT1 (UPB1) transcription factor plays a regulatory role in maintaining the balance between cell proliferation and differentiation, by controlling the expression of peroxidases and thus maintaining ROS homeostasis (Tsukagoshi et al., 2010).

3. Programmed cell death in plant development

PCD in plants shares common traits with animal cell apoptosis such as shrinkage of cytoplasm, condensation and aggregation of the chromatin and nuclear DNA cleavage. There are also features of PCD, which are unique to plants such as rigid cell walls and the absence of phagocytic cells. Furthermore, plant cells fail to form apoptotic bodies during PCD, which are distinctive for animal apoptosis (as reviewed by Van Doorn et al., 2011). PCD in plants can be divided into two broad categories, namely developmentally regulated and environmentally induced. Developmentally regulated PCD occurs at certain time points and locations. In contrast, environmentally induced PCD, such as the hypersensitive response (HR), is initiated by external biotic and abiotic signals.

3.1. PCD and embryo development

PCD plays an important role during embryo development. After fertilization, the zygote undergoes cell division giving rise to apical and basal cells. The apical cell forms the embryo, whereas the basal cell initiates to the suspensor. The suspensor is responsible for the transfer of nutrients from the endosperm to the embryo. After completion of this function, the PCD process eliminates the suspensor cells. PCD in suspensor cells involves metacaspase activity. The *mcII-Pa*, *Picea abies* cysteine protease metacaspase, is involved in the final steps of suspensor cells elimination. The *mcII-Pa* metacaspase translocates from cytoplasm to the nucleus, concentrating in the nuclear pore complex and chromatin, which results in

disintegration of the nuclear envelope and DNA breakage (Bozhkov et al., 2005). Furthermore, the *Arabidopsis kiss of death* (KOD) gene has also been reported to be involved in the activation of the PCD pathway in suspensor cells. The *kod-2* mutant plants exhibit suppression of the PCD process in the suspensor cells and in root hairs (Blanvillain et al., 2011).

3.2. PCD and seed germination

A plant seed is mainly the assembly of embryo and stored food covered by a seed coat. The stored food is necessary for the early development of the embryo before a root system is established and the photosynthetic capability is attained. During seed germination in cereal crops such as barley and rice, aleurone cells form a secretory tissue and *de novo* synthesis of hydrolytic enzymes takes place. These hydrolytic enzymes are used for the breakdown of the reserves stored as starch in the endosperm and utilized for seedling growth. The activity of α-amylase and other hydrolytic enzymes is regulated by Gibberellin (GA) in aleurone cells (Eastmond and Jones, 2005; Palma and Kermode, 2003). After the completion of digestive activity, aleurone cells are eliminated by the PCD process. During the onset of PCD, GA plays a positive role by regulating ROS levels in aleurone cells (Palma and Kermode, 2003).

3.3. PCD in tracheary element differentiation

The xylem tissues function as the main transporters of water from the source to the sink inside the plant body. Xylem consists of tracheary elements (TE), parenchyma cells and fiber cells. TEs serve as conductive tubes for water, and together with fiber cells, they provide mechanical support for the plant body. PCD plays a pivotal role during the development of TEs (Obara et al., 2001). The PCD process proceeds with a rapid collapse of the vacuole, leading to nuclear degradation and cell death. This is followed by complete degradation of cellular contents and thereby formation of hollow channels for conducting water to different plant tissues (Ito and Fukuda, 2002). Recently, it was reported that NAC (for NAM, ATAF-1, -2 and CUC2) domain proteins participate in the development of TEs by initiating

PCD. For example, up-regulation of genes involved in the PCD process was observed under the influence of VASCULAR-RELATED NAC-DOMAIN6 (VND6). In addition, VND6 also modulates the regulation of genes containing a TE-specific cis-element (TERE) in their promoters (Ohashi-Ito et al., 2010).

3.4. PCD in anther development

The anther is the male reproductive tissue of the plant containing haploid microspores also called pollen. It comprises meiotic cells surrounded by the anther wall. The tapetum, the innermost cellular layer of the anther, plays a crucial role in pollen formation. Thus, the development and differentiation of tapetum is important for the initial stage of male reproduction. In turn, the PCD-triggered degradation of tapetum is necessary for the development of viable pollen and especially for pollen maturation (Aya et al., 2009; Li et al., 2006). For example, failure in the activity of the *Oryza sativa* Persistent Tapetal Cell1 (PTC1) results in tapetal proliferation, abnormal pollen formation and male sterility, indicating a regulatory role for this protein in the PCD process taking place in tapetal cells (Li and Xing, 2011).

3.5. PCD and self-incompatibility

Self-incompatibility is a genetically controlled mechanism to prevent inbreeding in different plant species by rejecting incompatible pollen. For example, during the pollen-pistil interaction in *Papaver*, the pistil multi-allelic S locus recognizes the incompatible pollen and subsequently inhibits its growth. The pistil S-determinant locus releases signals that trigger PCD in the incompatible pollen upon the recognition of pollen tube growth (Geitmann et al., 2004). The *Papaver rhoeas* pollen S gene (*PrpS*) was isolated and shown to encode for the pollen S locus determinant. *PrpS* is involved in the S-specific inhibition of incompatible pollen (Wheeler et al., 2009). The proteins produced by the pistil S-locus trigger increase in the Calcium (Ca^{2+}) influx, Cytochrome c (*Cyt c*) and caspase 3-like activity in the dying cells of the pollen tube (Thomas and Franklin-Tong, 2004). The Ca^{2+} influx in turn stimulates the production of ROS and $NO^{\bullet}$ in the pollen tube. ROS and $NO^{\bullet}$ act

as signaling molecules, upstream of caspase 3-like activity, during the PCD process (Wilkins et al., 2011).

3.6. PCD during leaf development

PCD plays an important role during leaf development such as formation of trichomes, lace leaf shape and during leaf senescence. Trichomes are the epidermal outgrowths of many plant species, comprised of a single to many cells. Emerging trichomes stop mitotic cell divisions, increase in size and grow perpendicularly to the surface of the leaf. This is followed by cell branching, expansion and endoreduplication. After the completion of the developmental cycle, a destructive phase, induced by H_2O_2, starts within the trichome cells (as reviewed by Hulskamp, 2004).

Another unique function of PCD in plants is the development of complex leaf shape. In the lace plant (*Aponogeton madagascariensis*), during development the leaf blades form characteristic perforations through the death of distinct patches of cells. The dying cells at each perforation are subdued to PCD by the disruption of vacuoles, condensation of cytoplasm and fragmentation of the nuclear contents (Gunawardena et al., 2004).

3.7. PCD in leaf senescence

Leaf senescence is an age dependent developmental type of PCD in plants. It is a tightly regulated genetic process and is influenced by different phytohormones. During this phenomenon, healthy tissues undergo degradation or cell death, which leads to nutrient recovery to support metabolism of other organs. Thus, leaf senescence in plants is the terminal event that allows the reutilization of cellular material of the tissues and organs subjected to elimination by PCD. Apart from age dependent leaf senescence, biotic and abiotic stress conditions can also initiate early senescence in the leaf tissue. These stress conditions include pathogen attack, drought, extreme temperature and ozone, and have in common the induction of an oxidative burst (as reviewed by Lim et al., 2007). Ultimately, the ROS trigger PCD in the leaf cells. Some of the available delayed senescence mutants exhibited

tolerance to ROS, which is in agreement with the previous notion, at least in part, that ROS are involved in leaf senescence (Woo et al., 2004). Recent analysis identified a number of genes that participate in the regulation of leaf senescence. These genes are collectively known as senescence-associated genes (SAG) (Lim et al., 2007). One typical example Onset of Leaf Death3-1 (OLD3-1), encoding the cytosolic O-acetylserine(thiol)lyase in *Arabidopsis*. The *old3-1* mutant plants exhibited an early leaf senescence phenotype. The analysis of mutant plants provided a link between the OLD3-1 gene and cell death in relation to plant stress responses and defense mechanisms (Shirzadian-Khorramabad et al., 2010).

4. Abiotic stress induced PCD

Exposure of plants to abiotic stress factors such as high light conditions, ultraviolet-light, ozone (O_3), salinity, drought, extreme temperatures, pollutants like heavy metals, 3-aminotriazole (AT) and methyl-viologen (paraquat; PQ) can lead to oxidative stress and subsequently to ROS-dependent PCD. Under high light conditions, hyper-reduction of the electron transport chain results in photo-inactivation of PSII. Consequently, only PSI remains operational in order to prevent stress-induced inhibition of photosynthesis, which in turn causes over-production of H_2O_2. In these conditions, photo-inhibition is the reason for the accumulation of $^1O_2$ as well. Although $^1O_2$ has a short half-life, it is highly reactive. The cell death associated with $^1O_2$-induced oxidative damage is the result of a dynamic genetic process. In the conditional *flu* mutant, the generation of $^1O_2$ that takes place in plastids is boosted. This localized formation of $^1O_2$ can trigger a change in gene expression inside the nucleus by the activation of more stable second messengers in the plastids. As an example, plastid proteins such as EXECUTER1 (EX1) and EXECUTER2 (EX2) are involved in the signal transduction from plastids to the nucleus (Lee et al., 2007).

Another adverse condition that can trigger photo-oxidative stress in plants is the overexposure to UV-light. ROS production in mitochondria under UV-treatment acts as a signal that causes mitochondrial dysfunction and subsequent PCD. The cell death induced by UV can be delayed by pretreatment of protoplasts with ascorbic

acid or DCMU (an inhibitor of the photosynthetic electron transport) (Gao et al., 2008). The exposure to UV-light activates metacaspase 8 (AtMC8), which indicates that AtMC8 is associated with cell death triggered by UV-light and ROS (He et al., 2008).

Ozone (O_3) is a major air pollutant with many harmful effects to plants. Application of lower concentrations of O_3 can lead to reduction of photosynthesis and growth, and early senescence. Higher concentrations of O_3 cause programmed cell death, producing lesions, which resemble the HR response during plant-pathogen interactions. It has been shown that the mechanism of O_3 action involves an initial stimulation of plant hormones such as jasmonic acid, salicylic acid and ethylene, which then orchestrate oxidative stress signals (Overmyer et al., 2005; Tamaoki et al., 2003). Accumulation of $NO^{\bullet}$ also takes place during O_3 stress, suggesting a role for $NO^{\bullet}$ as a signaling molecule during O_3-induced PCD (Ahlfors et al., 2009). O_3 toxicity can also trigger the activation of anion channels, which creates an influx of Ca^{2+} into the cytoplasm from the extracellular spaces. Ca^{2+} influx and ROS stimulate the activation of a vacuole processing enzyme (VPE) resulting in the collapse of the vacuole and ultimately in cell death (Kadono et al., 2010).

Heavy metals such as cadmium (Cd) can induce ROS-mediated PCD. Exposure of tobacco cells to Cd stress leads to TUNEL-positive nuclei with chromatin condensation and an increase in the expression of Hsr203J, a PCD-related gene. Additionally, the accompanying significant increase in $NO^{\bullet}$ production indicates that $NO^{\bullet}$ has a positive role in Cd-induced PCD (Ma et al., 2010). Similarly, Cd stress induces senescence-like PCD in *Arabidopsis* suspension cultures (De Michele et al., 2009). Aluminium (Al), like Cd, can also cause PCD through perturbations in ROS homeostasis and mitochondrial dysfunction (Li and Xing, 2011; Yin et al., 2010). Upon entering into the mitochondria, Al inhibits the electron transport chain, resulting in the production of ROS. The outcome of these events is the rupture of the mitochondrial membrane and the release of *Cyt c* into the cytoplasm. *Cyt c* activates a caspase-3-like pathway, which is also related to PCD (Li and Xing, 2011). The introduction of the nematode apoptotic suppressor (Ced-9),

a Bcl-2 homologue, in tobacco prevents Al-induced PCD by inhibiting the caspase-like pathway and VPE activity via an unknown mechanism (Wang et al., 2009).

Herbicides such as AT and PQ have the ability to destroy plants by inducing ROS. AT inhibits the activity of catalase. As a result, accumulation of H_2O_2 takes place in plant cells, mainly in the peroxisomes. Higher concentrations of H_2O_2 can lead to cell death, whereas non-lethal concentrations of H_2O_2 act as signaling molecules. For example, pretreatment of tobacco plants with lower concentrations of H_2O_2 results in tolerance to AT induced oxidative stress. Pretreatment with H_2O_2 also raises the APX activity, which might prevent AT-induced oxidative damage (Gechev et al., 2002). In *cat* mutant plants, AT-application amplifies the activity of abscisic acid (ABA), which further triggers ROS production. As a result, elevation of H_2O_2 in particular provokes stomatal closure (Jannat et al., 2011). Microarray analysis of plants subjected to AT-mediated PCD in *Arabidopsis* shows an early regulation of H_2O_2-responsive genes including an oxoglutarate-dependent dioxygenase. A knockout of the oxoglutarate-dependent dioxygenase gene results in the reduction of cell death symptoms, indicating a role of this gene in the PCD machinery (Gechev et al., 2005).

PQ, a non-selective herbicide, is capable of inducing oxidative stress in chloroplasts by donating an electron to oxygen during photosynthesis, which produces O_2^-. The O_2^- is further transformed into H_2O_2. Plants can tolerate PQ-mediated stress either by the activity of antioxidants such as SOD and/or APX or by blocking the cell death-signaling pathway (Chen et al., 2009; Fujibe et al., 2004; Murgia et al., 2004). The *Arabidopsis* LAG one homologue2 (*loh2*) genotype, an *Alternaria alternata* f. sp. *lycopersici*-toxins (AAL) sensitive mutant, also exhibits susceptibility towards PQ treatment. The genetic screening of the progeny of *loh2* seeds treated with ethyl methanesulfonate (EMS) resulted in the isolation of nine *atr* mutants (AAL-toxin resistant; *atr1*-*atr9*). The fresh weight analysis of *loh2* and nine *atr* mutants under PQ-induced oxidative stress indicated that *atr* mutants are more tolerant when compared to *loh2*. However, variation in resistance to PQ was observed among the different *atr* mutants (Gechev et al., 2008; Qureshi et al., 2011). Identification and characterization of these *atr* mutations will help in the explanation

of PQ-induced PCD. The oxidative stress tolerant 6 (*oxt6*) exhibits increased tolerance to AT, PQ and BSO (buthionine S, R-sulfoximine; an inhibitor of glutathione synthesis). The analysis of *oxt6* indicated that the gene encodes the 30-kD subunit of cleavage and polyadenylation specificity factor (CPSF30). The transcriptome analysis and single gene expression studies of *oxt6* show the induction of genes encoding proteins containing thioredoxin- and glutaredoxin-associated domains. In addition, it was observed that the poly(A) site selection was different in the *oxt6* mutant as compared to wild type indicating the indirect effect of CPSF30 in polyadenylation (Zhang et al., 2008).

5. PCD in biotic stress

Fungal plant pathogens can be divided into two broad categories based on their interaction with their host: biotrophs and necrotrophs. Biotrophs feed on the living host through specialized structures, whereas necrotrophs specialize in destroying the cell by the release of host-selective toxins that trigger PCD at the site of infection and the pathogen feeds on the dead cells. Fungal pathogens such as *Fusarium* and *Alternaria* spp. produce toxins that perturb the sphingolipid biosynthesis pathway in plants, which ultimately results in PCD. *Fusarium moniliforme* and *Alternaria alternata* f. sp. *lycopersici* secrete fumonisin B_1 (FB_1) and AAL toxins in maize and tomato, respectively. These host-selective mycotoxins are analogous to plant sphinganine and are known as sphinganine-analog mycotoxins (SAM). Cell death triggered by FB_1 and AAL-toxins exhibits all the hallmarks of PCD, including nuclear condensation and fragmentation. VPE plays an important role in the FB_1-induced cell death process in plants (Kuroyanagi et al., 2005). Mutation of Bax inhibitor1 (*AtBI1*) results in accelerated cell death caused by FB_1 in *Arabidopsis*, indicating the role of *AtBI1* in FB_1-mediated PCD (Watanabe and Lam, 2008). Another necrotrophic fungus, *Botrytis cinerea*, triggers PCD in many plants, including *Arabidopsis*, by secreting a variety of low molecular weight phytotoxins like botrydial (Colmenares et al., 2002; Van Baarlen et al., 2004). This results in an oxidative burst and the accumulation of free radicals and H_2O_2 at the site of infection and surrounding areas (Schouten et al., 2002).

Plants use PCD also as a defense strategy against biotrophic pathogens. This phenomenon is known as the hypersensitive response (HR). HR defense responses are initiated in order to curtail the threatening pathogen at the site of infection and thus, restrict their movement. In the case of an avirulent pathogen, the defense process includes two steps. After penetration, the pathogen is detected by pathogen-associated molecular patterns (PAMP). PAMPs are recognized by trans-membrane receptors, which can detect pathogen molecules such as bacterial flagellin and fungal chitin (Kaku et al., 2006; Zipfel et al., 2004). Host-adapted pathogens are able to interfere in this basal plant immune system with a variety of effector molecules. Therefore, plants have developed a second line of defense called effector-mediated immunity (ETI) to counteract the effects of the pathogen effector molecules. The plant R proteins encoded by plant disease resistance R genes recognize the effector proteins. After recognition, plant R proteins initiate a signaling cascade that triggers the death of plant cells surrounding the site of infection, stomatal closure and induce systemic acquired resistance (SAR). SAR activates the defense machinery in distal plant parts in order to protect the undamaged tissues against the invading pathogen.

The HR type of PCD causes the influx of Ca^{2+} into the cytoplasm and activation of a mitogen-activated protein kinase (MAPK) signaling cascade, followed by the production of plant hormones, ROS and $NO^{\bullet}$ (Coll et al., 2011; Gechev et al., 2006; Torres et al., 2006). VPE is emerging as an important executioner of this type of PCD. It is a cysteine proteinase responsible for the activation of different vacuolar proteins and is involved in various forms of stress induced cell death (Nakaune et al., 2005; Rojo et al., 2004; Wang et al., 2009). VPE, which has caspase-1 like activity, mediates disruption of the vacuolar membrane, resulting in the release of vacuolar contents, including hydrolases, into the cytoplasm. The subsequent cell death is of the HR type. The importance of VPE as a key molecule in HR is illustrated by its suppression, which leads to the inhibition of hypersensitive type of PCD in plants (Hatsugai et al., 2004; Kuroyanagi et al., 2005). This strategy is functional against pathogens penetrating the cell. However, some pathogens, like avirulent strains of *Pseudomonas syringae*, do not enter the

plant host cells, but proliferate in the intercellular space. Plants use another strategy against these types of threats. In response to *P. syringae* infection, the large central vacuole fuses to the plasma membrane prior to cell death. This results in the discharge of vacuolar proteases that disrupts bacterial growth and causes HR cell death of the surrounding cells. The fusion of vacuole and plasma membrane is regulated by the proteasome (Hatsugai et al., 2009).

6. Sphingolipid metabolism in relation to PCD

Sphingolipids are structurally diverse molecules. They are lipids containing sphingoid bases and are essential components of the endomembrane system in eukaryotes. The structural components of plant sphingolipids consist of a long-chain base (LCB) with an amide linked to a fatty acid (FA). The synthesis of sphingolipids is carried out by N-acylation of the amino group of an LCB with a free FA, which acts as the acyl donor (figure 2). Plant sphingolipids can be divided into two major groups, namely glycosylceramides and inositol-phosphorylceramides. This classification is based upon the type of the head group attached to the LCB (as reviewed by Spassieva and Hille, 2003). Sphingolipids are components of the tonoplast, plasma membranes and microdomains or lipid rafts, which are involved in plasma membrane trafficking (Borner et al., 2005; Markham et al., 2011). In addition, sphingolipids play an important regulatory role in different cellular processes such as plant growth, ABA dependent guard cell closure and PCD (Chen et al., 2008; Ng et al., 2001; Shi et al., 2007; Spassieva et al., 2002; Wang et al., 2008).

Ceramide synthase is involved in the biosynthesis of complex ceramides from sphinganine. Inhibition of its enzymatic activity results in disruption of sphingolipid biosynthesis and accumulation of sphingoid bases. The depletion of complex ceramides and release of free sphingoid bases can lead to PCD (Brandwagt et al., 2000; Liang et al., 2003; Spassieva et al., 2002). Host-selective mycotoxins such as FB_1 and AAL toxins compromise sphingolipid biosynthesis by inhibiting the activity of ceramide synthase (Sphinganine N-acyltransferase). A single *Asc* gene (*Alternaria* stem canker) can inhibit AAL toxins-induced PCD in tomato. The *Asc*

gene is homologous to the yeast Longevity Assurance Gene1 (LAG1) (Brandwagt et al., 2000). Although, *Arabidopsis thaliana* is relatively insensitive to AAL-toxins treatment, a T-DNA knockout of a LAG1 homologue in *Arabidopsis* renders mutant plants sensitive to its action. This *Arabidopsis* knockout was called *loh2*. Infiltration of *loh2* leaves with 200 nM AAL-toxins induces cell death three days after application. Staining of the AAL-toxins treated *loh2* leaves with specific cytochemicals indicates an oxidative burst prior to visible cell death symptoms. Furthermore, transcriptional profiling of these leaves demonstrates up-regulation of genes associated with ROS and the ethylene-signaling pathway along with the down-regulation of genes previously associated with growth and development.

The precursors of ceramides are also bioactive molecules. For example, LCBs can also act as mediators of PCD. They are comprised of up to 18 carbon atoms (C-18) and up to three hydroxyl groups (Markham et al., 2006). Trihydroxy LCBs are the major components of plant sphingolipids and account for nearly 90% of the sphingolipids in the leaves of *Arabidopsis*. Complete loss of trihydroxy LCBs results in severe dwarfism due to defects in cell expansion and division. The trihydroxy LCB-deficient plants are also unable to shift from the vegetative to the reproductive phase. Moreover, double mutants of the LCB C-4 hydroxylase genes Sphingoid Base Hydroxylase1 (SBH1) and SBH2 exhibit necrotic lesions and premature death due to the buildup of sphingolipids, including free LCBs and ceramides (Chen et al., 2008). Interestingly, the LCB-Ps, sphingosine 1 phosphate, has the ability to inhibit PCD in *Arabidopsis* cell cultures subjected to thermal-stress (Alden et al., 2011). The synthesis of LCB is catalyzed by serine palmitoyl transferase (SPT), an ER-associated heteromeric protein. The enzyme catalyzes the formation of 3-keto dihydrosphingosine from L-serine and palmitoyl CoA. 3-ketosphinganine is then immediately converted to sphinganine. Two genes, LCB1 and LCB2, are essential for the SPT activity (Dietrich et al., 2008; Spassieva and Hille, 2003). LCB1 is thought to be involved in PCD as observed in the *Arabidopsis* mutant fumonisin B_1 resistant11-1 (*fbr11-1*) which is unaffected by FB_1 treatment. The mutant plants fail to build up ROS and display no obvious signs of PCD when treated with FB_1. Genetic screening demonstrated that FBR11 actually encodes

LCB1 (Shi et al., 2007). The function of LCB1 and LCB2 in the induction of PCD during male gametophyte development in *Arabidopsis* was also reported. *fbr11-2* plants, defective for an allele of the *Arabidopsis* LCB1 gene (*lcb1-1*), exhibit PCD symptoms that lead to abnormalities in the microspores. Moreover, mutation of LCB2 results in the same phenomenon, confirming that the SPT activity is essential for the normal development of the male gametophyte (Teng et al., 2008). In addition, LCB2 is required to support an effective PCD-induced defense strategy against pathogens. Overexpression of LCB2 in *Nicotiana benthamiana* provokes an HR-type of cell death during infection by non-host pathogens (Takahashi et al., 2009). The LCB2 gene possesses two copies in *Arabidopsis*, *AtLCB2a* and *AtLCB2b*. Knockout of both copies resulted in cell lethality indicating that sphingolipid synthesis is essential for plant cell viability (Dietrich et al., 2008). However, a recent study provided evidence that not the LCB but Very-Long-Chain Fatty Acid (VLCFA) sphingolipids (C > 18 carbons) are essential for plant

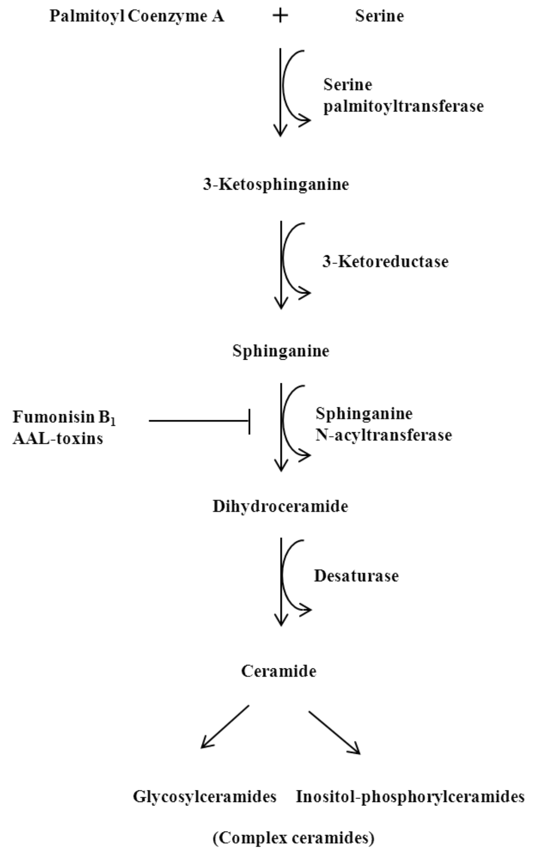


Figure 2: Schematic representation of sphingolipids biosynthesis in plants as described in the text. The fungal toxins fumonisin B_1 (FB_1) and *Alternaria alternata* f. sp. *lycopersici* (AAL-toxins) disrupt the formation of dihydroceramide by inhibiting the activity of the enzyme sphinganine N- acyltransferase.

development, as inability to synthesize VLCFA leads to lethality (Markham et al., 2011).

Conversion of ceramides into complex forms such as inositolphosphorylceramide (IPC) is catalyzed by IPC synthase that transfers phosphatidylinositol to ceramide. It was reported that IPC synthase acts as a regulator of plant sphingolipid metabolism and in pathogen resistance associated PCD. The ERH1 gene (Enhancing RPW8-mediated HR-like cell death1) encodes an IPC synthase in *Arabidopsis*. Loss of function of ERH1 results in dramatic accumulation of ceramides and a reduction of IPC. The *erh1* mutant plants exhibit enhanced HR-like cell death when challenged with the powdery mildew fungus. The results suggest that ERH1 functions as a negative regulator in pathogen-induced PCD (Wang et al., 2008).

All these novel findings confirm the regulatory role of sphingolipid metabolism in plant development, reproduction, immune system, control of ROS generation and cell death. Since the depletion of complex ceramides and accumulation of their precursors mainly leads to growth inhibition and PCD, a fine balance between complex ceramides and their precursors coordinates the cell survival. Together they seem to operate as a rheostat in the processes leading to PCD.

7. PCD signaling cascades in plants

ROS are molecules with dual nature. Apart from acting as a threat to the cell and its internal organelles, they can also serve as signaling molecules that can initiate defense mechanisms against biotic and abiotic stress. Many different components involved in the cell death-signaling network have been identified in the last few years. Despite these achievements, this network remains puzzling. Cell death stimuli such as salinity, drought and pathogen attack trigger the activation of the plasma membrane NADPH oxidase and a Ca^{2+} channel, which in turn induce ROS production and Ca^{2+} influx, respectively. Conversely, ROS are able to provoke the influx of Ca^{2+} after elicitation (Blume et al., 2000; Grant et al., 2000). Downstream in the signaling process, the high levels of ROS activate the MAPK

signaling cascade. As a result, a massive reprogramming of TFs takes place in the nucleus (figure 3).

In plants, cytosolic Ca^{2+} acts as a second messenger involved in various signaling cascades responding to biotic and abiotic stress stimuli. In the innate plant immune system such as the HR, the perception of signals from pathogen attack results in the elevation of cytosolic Ca^{2+} through the influx of extracellular calcium across Ca^{2+} channels. Cytosolic Ca^{2+} is associated with the activation of PCD in *Arabidopsis* through the regulation of ROS. It was suggested that Ca^{2+} influx functions upstream of ROS production during the HR response. The disruption of Ca^{2+} currents by a calcium-channel blocker represses the formation of H_2O_2 and progression of PCD (Grant et al., 2000). The increase in cytosolic Ca^{2+} is perceived by calcium sensors, which transduce the stimuli into appropriate physiological responses through interaction with downstream target proteins. This signaling network includes the activation of calcium dependent protein kinases (CDPK), calmodulins (CaM) and Calcineurin B-like proteins (CBL). CaMs and CaM-like proteins are a large family of Ca^{2+} binding proteins. Modification of CaMs by the increase of cytosolic Ca^{2+} facilitates their interaction with other proteins. *Arabidopsis* AtSR1, a CaM binding transcription factor, acts as a regulator of salicylic acid dependent resistance to pathogens (Du et al., 2009). CDPKs are serine/threonine protein kinases consisting of four conserved domains. Ca^{2+} influx triggers the activation of CDPKs through the deactivation of an auto-inhibitory domain and the activation of the C-terminal CaM-like domain. At basal levels of Ca^{2+} in the cells, CDPKs are inactivated by the auto-inhibitory domain. Under the influence of Ca^{2+} influx, the CDPKs bind to Ca^{2+} through a CaM-like domain, which displaces the auto-inhibitory domain and leads to the activation of CDPKs. The consequences are ROS accumulation and PCD (Ludwig et al., 2004; Wernimont et al., 2010).

The MAPK cascade is the major signaling pathway for transducing a variety of stimuli during plant growth and development or stress responses, including PCD. Plant MAPKs are a distinct class of serine/threonine protein kinases. In the *Arabidopsis* genome, the MAPK cascade consists of more than 20 MAPKs and 10

MAPKKs, 60 MAPKKKs, which can interact with one another in dependence on the environmental stimuli or developmental stage (Ichimura et al., 2002). The activation of the HR-specific MAPK cascade consisting of SIPK, Ntf4 and WIPK, triggers PCD in tobacco. It was also reported that direct activation of this MAPK cascade leads to light dependent disruption of photosynthetic activity and generation of ROS in chloroplasts (Liu et al., 2007). Another study shows that the oxidative burst by NADPH oxidase during plant-pathogen interactions is also mediated by MAPK activity in tobacco (Asai et al., 2008). ROS are also able to stimulate the MAPK signaling cascade, as observed in *Arabidopsis*. The application of ROS inducing agents to the leaves, such as AT and PQ, triggers the activation of AtMPK6 (Yuasa et al., 2001). The AtMPK6 in turn triggers the activation of the PCD pathway by disrupting sphingolipid metabolism (Saucedo-García et al., 2011).

NADPH oxidases are key enzymes involved in the production of ROS. Therefore, they play a crucial role in PCD through alterations in ROS homeostasis. The *Arabidopsis* genome contains ten AtRboh genes that encode NADPH oxidases. These genes are regulated either by Ca^{2+} or by CDPK signaling (Kobayashi et al., 2007; Torres and Dangl, 2005). Moreover, MAPKs also play a role in the regulation of Rboh genes in tobacco (Yoshioka et al., 2003).

A large fraction of senescence-associated genes (SAG) consists of NACs. NACs are senescence associated TFs and in *Arabidopsis*, more than 20 NAC TFs are expressed during this developmental stage. A NAC TF called ATAF1, was found to be induced by drought, salinity, ABA, methyl jasmonate, mechanical wounding and biotic stress (Wu et al., 2009). In *Arabidopsis*, two NAC TFs, ANAC029 (also called AtNAP) and ANAC092 (also known as AtNAC2 and ORE1) are considered as important regulators of senescence. Evidence indicates that ANAC029 acts as a positive modulator of senescence, as blocking its activity results in late senescence. Additionally, ANAC092 and ORS1 are thought to be involved in salinity induced senescence (Balazadeh et al., 2010; Balazadeh et al., 2011; Guo and Gan, 2006). It was observed that overexpression of ORS1, a controller of leaf senescence in *Arabidopsis thaliana*, resulted in the acceleration of leaf senescence in transgenic plants. On the other hand, silencing ORS1 leads to a delay in senescence.

Moreover, ORS1 triggers the activation of SAGs during long-term treatment with salt and H_2O_2 and subsequent enhancement of senescence in transgenic plants. It was established that cross-talk exists between ORS1 and H_2O_2-/salt-signaling pathways (Balazadeh et al., 2011).

The transcriptional reprogramming ultimately activates plant cell proteases. These proteases include VPE, which has a caspase-like activity, and metacaspases. They are involved in the final steps of PCD through their proteolytic activity. Although plants generally do not encode caspases like animals, a caspase-like activity for VPE has been reported. Plants contain a small gene family for another type of proteases known as metacaspases. Metacaspases are involved in PCD in both plants and fungi. The fungal genome possesses only one class of metacaspases. In plants however, there are two sub-classes of metacaspases, metacaspases I and II. The difference is due to the presence of a proline-/glutamine-rich N-terminal extension in MCI which is absent in MCII. In *Arabidopsis thaliana*, there are nine genes encoding metacaspases (AtMC1-9), three genes for AtMCI and six for AtMCII, respectively (Uren et al., 2000). There are indications for the involvement of metacaspases in ROS-induced PCD. The *Arabidopsis* metacaspase (AtMC8) activity is highly up-regulated during the oxidative burst caused by UVC, H_2O_2 and PQ. Radical-induced Cell Death1 (RCD1) mediates the up-regulation of AtMC8 and its loss of function results in reduction of PCD symptoms caused by UVC and H_2O_2 (He et al., 2008). Similarly, mutations of MCII genes cause the reduction of cell death symptoms in response to bacterial pathogens (Watanabe and Lam, 2011). Additionally, type I metacaspases such as AtMC1 and AtMC2 are considered antagonistic regulators of PCD. AtMC1 acts as a death enhancer, whereas AtMC2 as a death suppressor. It is interesting to note that the genetic alteration of these two metacaspases renders the plant insensitive to the HR cell death response (Coll et al., 2011).

Plant hormones such as ethylene, brassinosteroids (BR), abscisic acid (ABA), salicylic acid (SA), GA and jasmonic acid (JA) are involved not only in growth and development but also in plant defense. Along with other signaling molecules, they regulate the PCD and SAR responses in a complex manner. For example, ABA-

dependent H_2O_2 homeostasis is essential for stomatal closure (Desikan et al., 2008). During root development, a fine-tuning of H_2O_2 by GA signaling promotes the growth of roots and root hairs. GA is also responsible for the inactivation of nuclear localized DELLA proteins. Their name is derived from the presence of a conserved amino acids sequence in the N-terminal domain (Harberd, 2003). Accumulation of DELLA proteins results in the up-regulation of genes encoding antioxidant enzymes. Thus, GA modulates biotic and abiotic stress tolerance through the regulation of DELLA proteins (Achard et al., 2008). Another example, the barley protein HVA22, was previously known to be induced by ABA signaling. Recently, it was observed that HVA22 negatively regulates the activation of PCD triggered by GA-signaling in aleurone cells. Therefore, it was proposed that ABA plays an antagonistic role in GA-mediated PCD (Guo and Ho, 2008). Another case of hormone antagonistic interaction is the cross-talk between the SA and the JA response pathways. The SA pathway is switched on by biotrophic pathogens and suppresses the JA-mediated defense that is activated by caterpillars (Koornneef et al., 2008). SA, together with ethylene, also plays a role in the production of ROS in plants under stress (Gechev et al., 2006). Brassinosteroids are also capable of modulating the antioxidant defense mechanism by regulating the production of H_2O_2 in maize. Zhang et al., (2010) demonstrated that BRs could induce the accumulation of H_2O_2, which in turn activates ZmMPK5. In a positive feedback manner, ZmMPK5 further stimulates H_2O_2 production.

8. New players in plant signaling and PCD

The real number of genes and factors involved in PCD is probably much higher, than those implicated so far. Some of these genes/factors are initiators while others are executers in this complex process. A microarray analysis of the ROS/PCD sensitive mutant *loh2* treated with AAL-toxins indicated 81 genes that showed regulation at 7 hours after treatment. Some of the genes were highly induced, whereas others were repressed. Some of the TFs represented in the microarray data include WRKY, ZAT, Rboh, NAM and XTH, indicating their potential role in PCD (table 2) (Gechev et al., 2004).

The name WRKY is derived from a highly conserved 60 amino acid WRKY domain. The WRKY gene family consists of 74 members in *Arabidopsis* (http://www.mpiz-koeln.mpg.de/english/research/pmidpt/somssich/WRKY_Super family/Arabidopsis_WRKY_Superfamily/index.html). Studies demonstrate that different stress factors can lead to activation of WRKY TFs and ROS induced cell death. WRKY TFs also mediate ABA signaling in aleurone cells (Xie et al., 2005). As mentioned earlier, salinity stress can cause PCD in plant roots and WRKY TFs play an important role during this stress. Two *Arabidopsis* WRKY TFs, WRKY25 and WRKY33, are upregulated during salt stress. Further analysis revealed that overexpression of both genes results in tolerance to NaCl, but sensitivity towards ABA (Jiang and Deyholos, 2009). WRKY TFs have also been characterized as defense TFs. AtWRKY18, reported to have a role in defense and senescence, increases the resistance to bacterial pathogens in transgenic plants by activating R gene expression (Chen and Chen, 2002). AtWRKY6 is subjected to auto-regulation by suppression of its own promoter and acts as a regulator of transcription during leaf senescence and plant defense by targeting the SIRK and PR1 genes, respectively (Robatzek and Somssich, 2002). Additionally, AtWRKY6 mutant plants exhibit growth defects under boron deficiency, indicating that WRKY6 activity is important in decreasing the damage caused by boron starvation (Kasajima et al., 2010).

Zinc finger proteins (ZFP) play a crucial role in many cellular functions including regulation of other TFs, protein-protein interactions and binding to DNA, RNA or other proteins. A newly described rice ZFP TF, (DST; drought and salt tolerance) controls the stomatal closure by adjusting the H_2O_2 level. A mutation of DST triggers the stomatal closure due to the H_2O_2 accumulation in the guard cells, resulting in plant tolerance to drought and salt stress (Huang et al., 2009). An *Arabidopsis* representative of this family of TFs is Zinc *Arabidopsis thaliana*12 (ZAT12), which was found to respond to many abiotic stresses such as oxidative, osmotic, salinity, high light and temperature stresses (Davletova et al., 2005b). ZAT12 was also observed to be involved in the regulation of cytosolic ascorbate peroxidase1 activity and expression of WRKY25 and ZAT7 during

Table 1: Overview of ROS scavenging enzymes and antioxidants in plants.

Gene	Function	Reference
Senescence associated genes		
ATAF1	Involved in senescence, induced by various biotic and abiotic stresses	Wu et al., 2009
ANAC029	Involved in senescence	Guo and Gan, 2006
ANAC092	Induced by salinity, involved in senescence	Balazadeh et al., 2010
ORS1	Induced by salinity, involved in senescence	Balazadeh et al., 2011
Metacaspases		
AtMC1	Involved in the increase in the PCD process	Coll et al., 2011
AtMC2	Involved in the suppression of the PCD process	Coll et al., 2011
AtMC8	Up-regulated by ROS caused by UVC and PQ, involved in PCD	He et al., 2008
RCD1	Mediator of AtMC8 activity	He et al., 2008
Mitogen-activated protein kinase		
ZmMPK5	Involved in H_2O_2 production	Zhang et al., 2010a
AtMPK6	Involved in PCD processes triggered by disruption of sphingolipid metabolism	Saucedo-García et al., 2011
WRKY gene family		
AtWRKY6	Involved in leaf senescence and plant defense, regulation of SIPK and PR1 genes, tolerance against boron deficiency	Robatzek and Somssich, 2002; Kasajima et al., 2010

AtWRKY18	Activation of R genes, defense against bacterial pathogens and senescence	Chen and Chen, 2002
AtWRKY25	Induced by salt stress, involved in tolerance to salinity, sensitivity to ABA	Jiang and Deyholos, 2009
AtWRKY33	Induced by salt stress, involved in tolerance to salinity, sensitivity to ABA	Jiang and Deyholos, 2009
Zinc Finger transcription factors		
ZAT12	Involved in abiotic stress, regulation of peroxidase 1, WRKY25 and ZAT7	Davletova et al., 2005b; Rizhsky et al., 2004
DST	Regulation of H_2O_2, involved in stomatal closure, tolerance to drought and salinity	Huang et al., 2009
OsLSD1	Involved in the HR-like response, triggers R genes expression	Wang et al., 2005
Miscellaneous		
oxt6	Involved in ROS related stress tolerance. Encodes the proteins containing thioredoxin- and glutaredoxin- associated domains	Zhang et al., 2008
KOD	Involved in the PCD process in suspensor cells. Act upstream of caspase-like activity and interact with Ca^{2+} signaling	Blanvillain et al., 2011
GSNOR1/ HOT5/PAR2	Involved in $NO^{\bullet}$ and ROS related signaling. Act as an inhibitor of PCD triggered by O_2^{-}.	Chen et al., 2009; Feechan et al., 2005; Lee et al., 2008

The abbreviations are: mit, mitochondria; cyt, cytoplasm; chl, chloroplasts; per, peroxisomes; gly, glyoxysomes.

oxidative stress (Rizhsky et al., 2004). As ZAT12 participates in the regulation of ROS, antioxidant enzymes and stress-related transcripts, it might also play a role in the PCD signaling cascade. The rice zinc finger protein OsLSD1 acts as a negative regulator of PCD, while it enhances the differentiation of calluses. It has been shown that overexpression of OsLSD1 in transgenic tobacco enhanced the tolerance to FB_1.

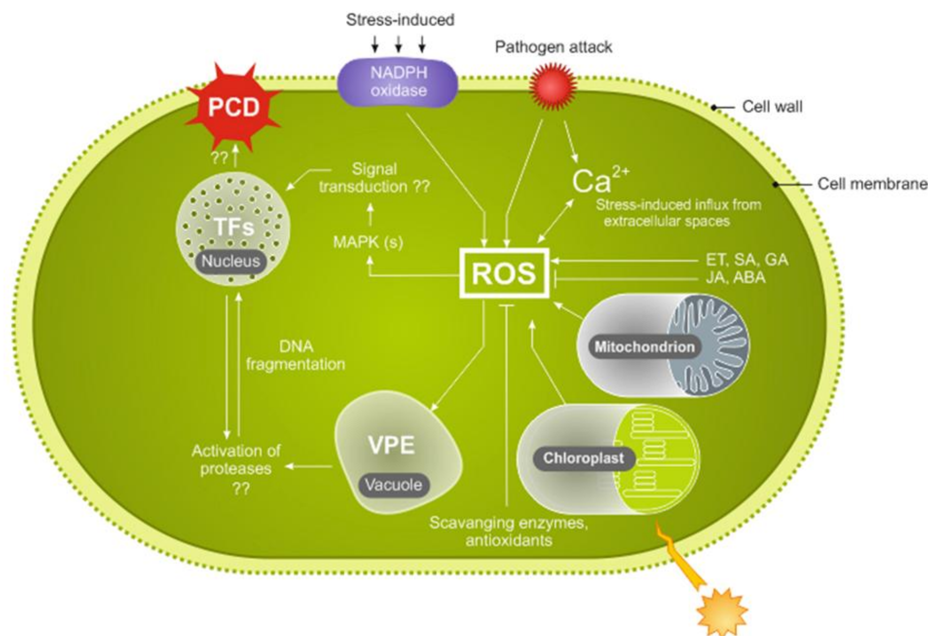


Figure 3: Programmed cell death in plants as described in the text. Reactive oxygen species (ROS) play a central role in the PCD process. The major ROS producing sites are the chloroplasts. Mitochondria and membrane bound NADP(H) oxidases also contribute in the ROS production under stress conditions. Antioxidant enzymes are involved in ROS detoxification. The cross-talk between various hormones such as ET, ethylene; SA, salicylic acid; GA, gibberellic acid; JA, jasmonic acid and ABA, abscisic acid dictates the ROS balance inside the cell. There is an antagonistic interaction between SA; JA and ABA; GA. The interaction between JA and ET is synergistic in the majority of cases. Under conditions favouring ROS production, there is an influx of Ca^{2+} from the extracellular space. Conversely, Ca^{2+} influx triggered by stress conditions favours ROS production. ROS activates VPE, a vacuole-processing enzyme, proteases and MAPK (s); mitogen-activated protein kinase (s), which subsequently activates PCD, related TFs, transcription factors inside the nucleus. The massive reprogramming ultimately leads to PCD.

Additionally, antisense OsLSD1 plants develop a lesion mimic phenotype and exhibit an HR-like response to the blast fungus (*Magnaporthe grisea*), with an increased expression of R genes (Wang et al., 2005).

The HR response is characterized by the accumulation of ROS, signaling molecules like $NO^{\bullet}$, SA and defense related genes. A fine balance between intracellular $NO^{\bullet}$ and ROS levels is important for the HR type of PCD (Delledonne et al., 2001). The major source of $NO^{\bullet}$ inside the cell is S-nitrosoglutathione (GSNO), which is derived by S-nitrosylation of the tripeptide glutathione. GSNO reductase (GSNOR) is the key enzyme involved in the reduction process. A mutation of *AtGSNOR1* resulted in the enhancement of S-nitrosylation, resulting in the interruption of the plant defense response. Conversely, increased activity of *AtGSNOR1* leads to reduction of S-nitrosylation levels and increased levels of GSNO. These results indicated a positive role of GSNOR in the plant defense reaction (Feechan et al., 2005). Recently, two genes identified in *Arabidopsis*, *sensitive to hot temperatures5* (*HOT5*) and PARAQUAT RESISRANT2 (*PAR2*), were found to be allelic to *GSNOR1*. The sensitivity to heat in *HOT5* mutant plants was associated with increased levels of $NO^{\bullet}$, indicating a role of GSNOR during abiotic stress signaling (Lee et al., 2008). The mutation in *PAR2* (*par2-1*) resulted in reduction of cell death symptoms when treated with PQ although the level of O_2^- accumulation was similar to that of wild type. It was postulated that *PAR2* might act in the PCD pathway induced by PQ as the *par2-1* mutation inhibited the signaling pathway triggered by PQ induced accumulation of O_2^- (Chen et al., 2009).

References

1. Achard P, Renou JP, Berthome R, Harberd NP, Genschik P (2008) Plant DELLAs restrain growth and promote survival of adversity by reducing the levels of reactive oxygen species. Curr Biol **18:** 656-660.
2. Ahlfors R, Brosche M, Kollist H, Kangasjarvi J (2009) Nitric oxide modulates ozone-induced cell death, hormone biosynthesis and gene expression in *Arabidopsis thaliana*. Plant J **58:** 1-12.
3. Alden KP, Dhondt-Cordelier S, McDonald KL, Reape TJ, Ng CK, McCabe PF, Leaver CJ (2011) Sphingolipid long chain base phosphates can regulate

apoptotic-like programmed cell death in plants. Biochem Biophys Res Commun **410:** 574-80.

4. Apel K, Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55:** 373-399.
5. Asai S, Ohta K, Yoshioka H (2008) MAPK signaling regulates nitric oxide and NADPH oxidase-dependent oxidative bursts in *Nicotiana benthamiana*. Plant Cell **20:** 1390-1406.
6. Aya K, Ueguchi-Tanaka M, Kondo M, Hamada K, Yano K, Nishimura M, Matsuoka M (2009) Gibberellin modulates anther development in rice via the transcriptional regulation of GAMYB. Plant Cell **21:** 1453-1472.
7. Balazadeh S, Siddiqui H, Allu AD, Matallana-Ramirez LP, Caldana C, Mehrnia M, Zanor MI, Kohler B, Mueller-Roeber B (2010) A gene regulatory network controlled by the NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. Plant J **62:** 250-264.
8. Balazadeh S, Kwasniewski M, Caldana C, Mehrnia M, Zanor MI, Xue G, Mueller-Roeber B (2011) ORS1, an H_2O_2-responsive NAC transcription factor, controls senescence in *Arabidopsis thaliana*. Mol Plant **4:** 346-360.
9. Blanvillain R, Young B, Cai YM, Hecht V, Varoquaux F, Delorme V, Lancelin JM, Delseny M, Gallois P (2011) The *Arabidopsis* peptide kiss of death is an inducer of programmed cell death. EMBO J **30:** 1173-1183.
10. Blume B, Nürnberger T, Nass N, Scheel D (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell **12:** 1425-1440.
11. Borner GH, Sherrier DJ, Weimar T, Michaelson LV, Hawkins ND, Macaskill A, Napier JA, Beale MH, Lilley KS, Dupree P (2005) Analysis of detergent-resistant membranes in *Arabidopsis*. Evidence for plasma membrane lipid rafts. Plant Physiol **137:** 104-116.
12. Bozhkov PV, Suarez MF, Filonova LH, Daniel G, Zamyatnin AA Jr, Rodriguez-Nieto S, Zhivotovsky B, Smertenko A (2005) Cysteine protease *mcII-Pa* executes programmed cell death during plant embryogenesis. Proc Natl Acad Sci U SA **102:** 14463-14468.
13. Brandwagt BF, Mesbah LA, Takken FLW, Laurent PL, Kneppers TJA, Hille J, Nijkamp HJJ (2000) A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp *lycopersici* toxins and fumonisin B1. Proc Natl Acad Sci USA **97:** 4961-4966.
14. Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ (2006) ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. Plant J **45:** 113-122.

15. Chen C, Chen Z (2002) Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen induced *Arabidopsis* transcription factor. Plant Physiol **129:** 706-716.
16. Chen M, Markham JE, Dietrich CR, Jaworski JG, Cahoon EB (2008) Sphingolipid long-chain base hydroxylation is important for growth and regulation of sphingolipid content and composition in *Arabidopsis*. Plant Cell **20:** 1862-1878.
17. Chen R, Sun S, Wang C, Li Y, Liang Y, An F, Li C, Dong H, Yang X, Zhang J, Zuo J (2009) The *Arabidopsis* PARAQUAT RESISTANT2 gene encodes an S-nitrosoglutathione reductase that is a key regulator of cell death. Cell Res **19:** 1377-1387.
18. Coll NS, Epple P, Dangl JL (2011) Programmed cell death in the plant immune system. Cell Death Differ **18:** 1-10.
19. Colmenares AJ, Aleu J, Durán-Patrón R, Collado IG, Hernández-Galán R (2002) The putative role of botrydial and related metabolites in the infection mechanism of *Botrytis cinerea*. J Chem Ecol **28:** 997-1005.
20. Dat J, Vandenabeele S, Vranová E, Van Montagu M, Inzé D, Van Breusegem F (2000) Dual action of the active oxygen species during plant stress responses. Cell Mol Life Sci **57:** 779-795.
21. Davletova S, Rizhsky L, Liang HJ, Zhong SQ, Oliver DJ, Coutu J, Shulaev V, Schlauch K, Mittler R (2005a) Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. Plant Cell **17:** 268-281.
22. Davletova S, Schlauch K, Coutu J, Mittler R (2005b) The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in *Arabidopsis*. Plant Physiol **139:** 847-856.
23. De Michele R, Vurro E, Rigo C, Costa A, Elviri L, Di Valentin M, Careri M, Zottini M, di Toppi LS, Lo Schiavo F (2009) Nitric oxide is involved in cadmium-induced programmed cell death in *Arabidopsis* suspension cultures. Plant Physiol **150:** 217-228.
24. DellaPenna D, Pogson BJ (2006) Vitamin synthesis in plants: tocopherols and carotenoids. Annu Rev Plant Biol **57:** 711-738.
25. Delledonne M, Zeier J, Marocco A, Lamb C (2001) Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc Natl Acad Sci USA **98:** 13454-13459.
26. Desikan R, Horak J, Chaban C, Mira-Rodado V, Witthoft J, Elgass K, Grefen C, Cheung MK, Meixner AJ, Hooley R, Neill SJ, Hancock JT, Harter K (2008) The histidine kinase AHK5 integrates endogenous and environmental signals in Arabidopsis guard cells. PloS One **3:** e2491.

27. Dietrich CR, Han G, Chen M, Howard berg R, Dunn TM, Cahoon EB (2008) Loss-of-function mutations and inducible RNAi suppression of *Arabidopsis* LCB2 genes reveal the critical role of sphingolipids in gametophytic and sporophytic cell viability. Plant J **54:** 284-298.
28. Dietz KJ (2003) Plant peroxiredoxins. Annu Rev Plant Biol **54:** 93-107.
29. Du L, Ali GS, Simons KA, Hou J, Yang T, Reddy AS, Poovaiah BW (2009) Ca^{2+}/metacaspase regulates salicylic-acid-mediated plant immunity. Nature **457:** 1154-1158.
30. Feechan A, Kwon E, Yun BW, Wang Y, Pallas JA, Loake GJ (2005) A central role for S-nitrosothiols in plant disease resistance. Proc Natl Acad Sci USA **102:** 8054-8059.
31. Fujibe T, Saji H, Arakawa K, (2004) A methyl viologen-resistant mutant of *Arabidopsis*, which is allelic to ozone-sensitive *rcd*1, is tolerant to supplemental ultraviolet-B irradiation. Plant Physiol **134:** 275-285.
32. Eastmond PJ, Jones RL (2005) Hormonal regulation of gluconeogenesis in cereal aleurone is strongly cultivar-dependent and gibberellin action involves SLENDER1 but not GAMYB. Plant J **44:** 483-493.
33. Gao A, Xing D, Li L, Zhang L (2008) Implication of reactive oxygen species and mitochondrial dysfunction in the early stages of plant programmed cell death induced by ultraviolet-C overexposure. Planta **227:** 755-767.
34. Gechev T, Gadjev I, Van Breusegem F, Inze D, Dukiandjiev S, Toneva V, Minkov I (2002) Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. Cell Mol Life Sci **59:** 708-714.
35. Gechev TS, Gadjev IZ, Hille J (2004) An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity of programmed cell death in plants. Cell Mol Life Sci **61:** 1185-1197.
36. Gechev TS, Hille J (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol **168:** 17-20.
37. Gechev TS, Minkov IN, Hille J (2005) Hydrogen peroxide-induced cell death in *Arabidopsis*: Transcriptional and mutant analysis reveals a role of an oxoglutarate-dependent dioxygenase gene in the cell death process. IUBMB Life **57:** 181-188.
38. Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays **28:** 1091-1101.
39. Gechev TS, Ferwerda MA, Mehterov N, Laloi C, Qureshi MK, Hille J (2008) *Arabidopsis* AAL-toxin-resistant mutant *atr1* shows enhanced tolerance to programmed cell death induced by reactive oxygen species. Biochem Biophys Res Commun **375:** 639-644.

40. Geitmann A, Franklin-Tong VE, Emons AC (2004) The self-incompatibility response in *Papaver rhoeas* pollen causes early and striking alterations to organelles. Cell Death Differ **11:** 812-822.
41. Gunawardena AHLA, Greenwood JS, Dengler NG (2004) Programmed cell death remodels lace plant leaf shape during development. Plant Cell **16:** 60-73.
42. Guo YF, Gan SS (2006) AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J **46:** 601-612.
43. Guo WJ, Ho TH (2008) An abscisic acid-induced protein, HVA22, inhibits gibberellin-mediated programmed cell death in cereal aleurone cells. Plant Physiol **147:** 1710-1722.
44. Grant M, Brown I, Adams S, Knight M, Ainslie A, Mansfield J (2000) The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J **23:** 441-450.
45. Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol **141:** 312-322.
46. Harberd NP (2003) Relieving DELLA restraint. Science **299:** 1853-1854.
47. Hatsugai N, Kuroyanagi M, Yamada K, Meshi T, Tsuda S, Kondo M, Nishimura M, Hara-Nishimura I (2004) A plant vacuolar protease, VPE, mediates virus-induced hypersensitive cell death. Science **305:** 855-858.
48. Hatsugai N, Iwasaki S, Tamura K, Kondo M, Fuji K, Ogasawara K (2009) A novel membrane fusion-mediated plant immunity against bacterial pathogens. Genes Dev **23:** 2496-2506.
49. He, R, Drury GE, Rotari VI, Gordon A, Willer M, Farzaneh T, Woltering EJ, Gallois P (2008) Metacaspase-8 modulates programmed cell death induced by ultraviolet light and H_2O_2 in *Arabidopsis*. J Biol Chem **283:** 774-783.
50. Hirayama T, Shinozaki K (2007) Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. Trends Plant Sci **12:** 343-351.
51. Huang XY, Chao DY, Gao JP, Zhu MZ, Shi M, Lin HX (2009) A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. Genes Dev **23:** 1805-1817.
52. Hulskamp M (2004) Plant trichomes: a model for cell differentiation. Nat Rev Mol Cell Biol **5:** 471-480.
53. Ichimura K, Shinozaki K, Tena G, Sheen J, Henry Y, Champion A, Kreis M, Zhang SQ, Hirt H, Wilson C, Heberle-Bors E, Ellis BE, Morris PC, Innes RW, Ecker JR, Scheel D, Klessig DF, Machida Y, Mundy J, Ohashi Y, Walker JC (2002) Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci **7:** 301-308.

54. Ito J, Fukuda H (2002) ZEN1 is a key enzyme in the degradation of nuclear DNA during programmed cell death of tracheary elements. Plant Cell **14:** 3201-3211.
55. Jannat R, Uraji M, Morofuji M, Islam MM, Bloom RE, Nakamura Y, McClung CR, Schroeder JI, Mori IC, Murata Y (2011) Roles of intracellular hydrogen peroxide accumulation in abscisic acid signaling in *Arabidopsis* guard cells. J Plant Physiol **168:** 1919-1926.
56. Jiang Y, Deyholos MK (2009) Functional characterization of *Arabidopsis* NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. Plant Mol Biol **69:** 91-105.
57. Kadono T, Tran D, Errakhi R, Hiramatsu T, Meimoun P, Briand J, Iwaya-Inoue M, Kawano T, Bouteau F (2010) Increased anion channel activity is an unavoidable event in ozone-induced programmed cell death. PloS One. **5:** e13373.
58. Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K (2006) Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. Proc Natl Acad Sci USA **103:** 11086-11091.
59. Kangasjarvi S, Lepisto A, Hannikainen K, Piippo M, Luomala EM, Aro EM, Rintamaki E (2008) Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. Biochem J **412:** 275-285.
60. Kasajima I, Ide Y, Yokota Hirai M, Fujiwara T (2010) WRKY6 is involved in the response to boron deficiency in *Arabidopsis thaliana*. Physiol Plant **139:** 80-92.
61. Kobayashi M, Ohura I, Kawakita K, Yokota N, Fujiwara M, Shimamoto K, Doke N, Yoshioka H (2007) Calcium-dependent protein kinases regulate the production of reactive oxygen species by potato NADPH oxidase. Plant Cell **19:** 1065-1080.
62. Koornneef A, Leon-Reyes A, Ritsema T, Verhage A, Den Otter FC, Van Loon LC, Pieterse CMJ (2008) Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. Plant Physiol **147:** 1358-1368.
63. Koppenol WH (2001) The Haber-Weiss cycle – 70 years later. Redox Rep **6:** 229-234.
64. Krieger-Liszkay A, Fufezan C, Trebst A (2008) Singlet oxygen production in photosystem II and related protection mechanism. Photosynth Res **98:** 551-564.
65. Kuroyanagi M, Yamada K, Hatsugai N, Kondo M, Nishimura M, Hara-Nishimura I (2005) VPE is essential for mycotoxin-induced cell death in *Arabidopsis thaliana*. J Biol Chem **280:** 32914-32920.

66. Lachaud C, Da Silva D, Amelot N, Béziat C, Brière C, Cotelle V, Graziana A, Grat S, Mazars C, Thuleau P (2010) Dihydrosphingosine-induced programmed cell death in tobacco BY-2 cells is independent of H_2O_2 production. Mol Plant **4:** 310-318.
67. Lee KP, Kim C, Landgraf F, Apel K (2007) EXECUTER1- and EXECUTER2-dependent transfer of stress-related signals from the plastid to the nucleus of *Arabidopsis thaliana*. Proc Natl Acad Sci USA **104:** 10270-10275.
68. Lee U, Wie C, Fernandez BO, Feelisch M, Vierling E (2008) Modulation of nitrosative stress by S-nitrosoglutathione reductase is critical for thermotolerance and plant growth in *Arabidopsis*. Plant Cell **20:** 786-802.
69. Li N, Zhang DS, Liu HS, Yin CS, Li XX, Liang WQ, Yuan Z, Xu B, Chu HW, Wang J, Wen TQ, Huang H, Luo D, Ma H, Zhang DB (2006) The rice tapetum degeneration retardation gene is required for tapetum degradation and anther development. Plant Cell **18:** 2999-3014.
70. Li Z, Xing D (2011) Mechanistic study of mitochondria-dependent programmed cell death induced by aluminium phytotoxicity using fluorescence techniques. J Exp Bot **62:** 331-343.
71. Liang H, Yao N, Song JT, Luo S, Lu H, Greenberg JT (2003) Ceramides modulate programmed cell death in plants. Genes Dev **17:** 2636-2641.
72. Lim PO, Kim HJ, Nam HG (2007) Leaf Senescence. Annu Rev Plant Biol **58:** 115-136.
73. Liu Y, Ren D, Pike S, Pallardy S, Gassmann W, Zhang S (2007) Chloroplast-generated reactive oxygen species are involved in hypersensitive response-like cell death mediated by a mitogen-activated protein kinase cascade. Plant J **51:** 941-954.
74. Ludwig AA, Romeis T, Jones JD (2004) CDPK-mediated metacaspase pathways: specificity and cross-talk. J Exp Bot **55:** 181-188.
75. Ma W, Xu W, Xu H, Chen Y, He Z, Ma M (2010) Nitric oxide modulates cadmium influx during cadmium-induced programmed cell death in tobacco BY-2 cells. Planta **232:** 325-335.
76. Markham JE, Li J, Cahoon EB, Jaworski JG (2006) Separation and identification of major plant sphingolipid classes from leaves. J Biol Chem **281:** 22684-22694.
77. Markham JE, Molino D, Gissot L, Bellec Y, Hématy K, Marion J, Belcram K, Palauqui JC, Satiat-Jeunemaître B, Faure JD (2011) Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in *Arabidopsis*. Plant Cell **23:** 2362-2378.
78. Mhamdi A, Hager J, Chaouch S, Queval G, Han Y, Taconnat L, Saindrenan P, Gouia H, Issakidis-Bourguet E, Renou JP, Noctor G (2010) *Arabidopsis* GLUTATHIONE REDUCTASE1 plays a crucial role in leaf responses to

intracellular hydrogen peroxide and in ensuring appropriate gene expression through both salicylic acid and jasmonic acid signaling pathways. Plant Physiol **153:** 1144-1160.
79. Mittler R, Vanderauwera S, Gollery M, Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci **9:** 490-498.
80. Murgia I, Tarantino D, Vannini C (2004) *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to paraquat-induced photooxidative stress and to nitric oxide-induced cell death. Plant J **38:** 940-953.
81. Nakaune S, Yamada K, Kondo M, Kato T, Tabata S, Nishimura M, Hara-Nishimura I (2005) A vacuolar processing enzyme, delta VPE, is involved in seed coat formation at the early stage of seed development. Plant Cell **17:** 876-887.
82. Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I (2008) Nitric oxide, stomatal closure, and abiotic stress. J Exp Bot **59:** 165-176.
83. Ng C, Carr K, McAinsh MR, Powell B, Hetherington AM (2001) Drought-induced guard cell signal transduction involves shpingosine-1-phosphate. Nature **410:** 596-599.
84. Obara K, Kuriyama H, Fukuda H (2001) Direct evidence of active and rapid nuclear degradation triggered by vacuole rupture during programmed cell death in Zinnia. Plant Physiol **125:** 615-626.
85. Ohashi-Ito K, Oda Y, Fukuda H (2010) *Arabidopsis* VASCULAR-RELATED NAC-DOMAIN6 directly regulates the genes that govern programmed cell death and secondary wall formation during xylem differentiation. Plant Cell **22:** 3461-3473.
86. Overmyer K, Brosche M, Pellinen R, Kuittinen T, Tuominen H (2005) Ozone-induced programmed cell death in the *Arabidopsis* radical-induced cell death1 mutant. Plant Physiol **137:** 1092-1104.
87. Palma K, Kermode AR (2003) Metabolism of hydrogen peroxide during reserve mobilization and programmed cell death of barley (*Hordeum vulgare* L.) aleurone layer cells. Free Radic Biol Med **35:** 1261-1270.
88. Pnueli L, Liang H, Rozenberg M, Mittler R (2003) Growth suppression, altered stomatal responses, and augmented induction of heat shock proteins in cytosolic ascorbate peroxidase (Apx1)-deficient *Arabidopsis* plants. Plant J **34:** 185-201.
89. Qureshi MK, Radeva V, Genkov T, Minkov I, Hille J, Gechev TS (2011) Isolation and characterization of *Arabidopsis* mutants with enhanced tolerance to oxidative stress. Acta Physiol Plant **33:** 375-382.

90. Rizhsky L, Davletova S, Liang H, Mittler R (2004) The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. J Biol Chem **279:** 11736-11743.
91. Robatzek S, Somssich IE (2002) Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev **16:** 1139-1149.
92. Rojo E, Martin R, Carter C, Zouhar J, Pan SQ, Plotnikova J, Jin HL, Paneque M, Sanchez-Serrano JJ, Baker B, Ausubel FM, Raikhel NV (2004) VPE gamma exhibits a caspase-like activity that contributes to defense against pathogens. Curr Biol **14:** 1897-1906.
93. Saucedo-García M, Guevara-García A, González-Solís A, Cruz-García F, Vázquez-Santana S, Markham JE, Lozano-Rosas MG, Dietrich CR, Ramos-Vega M, Cahoon EB, Gavilanes-Ruíz M (2011) MPK6, sphinganine and the LCB2a gene from serine palmitoyltransferase are required in the signaling pathway that mediates cell death induced by long chain bases in *Arabidopsis*. New Phytol **191:** 943-957.
94. Schouten A, Tenberge KB, Vermeer J, Stewart J, Wagemakers L, Williamson B, Van Kan JA (2002) Functional analysis of an extracellular catalase of *Botrytis cinerea*. Mol Plant Pathol **3:** 227-238.
95. Shi L, Bielawski J, Mu J, Dong H, Teng C, Zhang J, Yang X, Tomishige N, Hanada K, Hannun YA, Zuo J (2007) Involvement of sphingoid bases in mediating reactive oxygen intermediate production and programmed cell death in *Arabidopsis*. Cell Res **17:** 1030-1040.
96. Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol **6:** 410-417.
97. Shinozaki K, Yamaguchi-Shinozaki K (2007) Gene networks involved in drought stress response and tolerance. J Exp Bot **58:** 221-227.
98. Shirzadian-Khorramabad R, Jing HC, Everts GE, Schippers JH, Hille J, Dijkwel PP (2010) A mutation in the cytosolic O-acetylserine (thiol) lyase induces a genome-dependent early leaf death phenotype in *Arabidopsis*. BMC Plant Biol **10:** 80.
99. Smykowski A, Zimmermann P, Zentgraf U (2010) G-Box binding factor1 reduces CATALASE2 expression and regulates the onset of leaf senescence in *Arabidopsis*. Plant Physiol **153:** 1321-1331.
100. Snyrychova I, Pospisil P, Naus J (2006) Reaction pathways involved in the production of hydroxyl radicals in thylakoid membrane: EPR spin-trapping study. Photochem Photobiol Sci **5:** 472-476.
101. Spassieva SD, Markham JE, Hille J (2002) The plant disease resistance gene Asc-1 prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death. Plant J **32:** 561-572.

102. Spassieva S, Hille J (2003) Plant sphingolipids today - are they still enigmatic? Plant Biol **5:** 125-136.
103. Takahashi Y, Berberich T, Kanzaki H, Matsumura H, Saitoh H, Kusano T, Terauchi R (2009) Serine palmitoyltransferase, the first step enzyme in sphingolipid biosynthesis, is involved in nonhost resistance. Mol Plant Microbe Interact **22:** 31-38.
104. Tamaoki M, Matsuyama T, Kanna M, Nakajima N, Kubo A (2003) Differential ozone sensitivity among *Arabidopsis* accessions and its relevance to ethylene synthesis. Planta **216:** 552-560.
105. Teng C, Dong H, Shi L, Deng Y, Mu J, Zhang J, Yang X, Zuo J (2008) Serine palmitoyltransferase, a key enzyme for de novo synthesis of sphingolipids, is essential for male gametophyte development in *Arabidopsis*. Plant Physiol **146:** 1322-1332.
106. Thirkettle-Watts D, McCabe TC, Clifton R, Moore C, Finnegan PM, Day DA,Whelan J (2003) Analysis of the alternative oxidase promoters from soybean. Plant Physiol **133:** 1158-1169.
107. Thomas SG, Franklin-Tong VE (2004) Self-incompatibility triggers programmed cell death in Papaver pollen. Nature **429:** 305-309.
108. Torres MA, Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol **8:** 397-403.
109. Torres MA, Jones JDG, Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. Plant Physiol **141:** 373-378.
110. Tsukagoshi H, Busch W, Benfey PN (2010) Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell **143:** 606-616.
111. Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM (2000) Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell **6:** 961-967.
112. Van Baarlen P, Staats M, Van Kan JA (2004) Induction of programmed cell death in lily by the fungal pathogen *Botrytis elliptica*. Mol. Plant Pathol **5:** 559-574.
113. Van Breusegem F, Dat J (2006) Reactive oxygen species in plant cell death. Plant Physiol **141:** 384-390.
114. Van Doorn WG (2011) Classes of programmed cell death in plants, compared to those in animals. J Exp Bot **62:** 4749-4761.
115. Wang L, Pei Z, Tian Y, He C (2005) OsLSD1, a rice zinc finger protein, regulates programmed cell death and callus differentiation. Mol Plant Microbe Interact **18:** 375-384.

116. Wang W, Yang X, Tangchaiburana S, Ndeh R, Markham JE, Tsegaye Y, Dunn TM, Wang GL, Bellizi M, Parsons JF (2008) An inositolphosphorylceramide synthase is involved in regulation of plant programmed cell death associated with defense in *Arabidopsis*. Plant Cell **20:** 3163-3179.
117. Wang W, Pan J, Zheng K, Chen H, Shao H, Guo Y, Bian H, Han N, Wang J, Zhu M (2009) Ced-9 inhibits Al-induced programmed cell death and promotes Al tolerance in tobacco. Biochem Biophys Res Commun **383:** 141-145.
118. Watanabe N, Lam E (2008) BAX inhibitor-1 modulates endoplasmic reticulum stress-mediated programmed cell death in *Arabidopsis*. J Biol Chem **283:** 3200-3210.
119. Watanabe N, Lam E (2011) *Arabidopsis* metacaspases 2d is a positive mediator of cell death induced during biotic and abiotic stresses. Plant J **66:** 969-982.
120. Wernimont AK, Artz JD, Finerty P Jr, Lin YH, Amani M, Allali-Hassani A, (2010) Structures of apicomplexan calcium-dependent protein kinases reveal mechanism of activation by calcium. Nat Struct Mol Biol **17:** 596-601.
121. Wheeler MJ, de Graaf BH, Hadjiosif N, Perry RM, Poulter NS, Osman K, Vatovec S, Harper A, Franklin FC, Franklin-Tong VE (2009) Identification of the pollen self-incompatibility determinant in *Papaver rhoeas*. Nature **459:** 992-995.
122. Wilkins KA, Bancroft J, Bosch M, Ings J, Smirnoff N, Franklin-Tong VE (2011) Reactive oxygen species and nitric oxide mediate actin reorganization and programmed cell death in the self-incompatibility response of *Papaver*. Plant Physiol **156:** 404-416.
123. Woo HR, Kim JH, Nam HG, Lim PO (2004) The delayed leaf senescence mutants of *Arabidopsis*, *ore1*, *ore3*, and *ore9* are tolerant to oxidative stress. Plant Cell Physiol **45:** 923-932.
124. Wu YR, Deng ZY, Lai JB, Zhang YY, Yang CP, Yin BJ, Zhao QZ, Zhang L, Li Y, Yang CW, Xie Q (2009) Dual function of *Arabidopsis* ATAF1 in abiotic and biotic stress responses. Cell Res **19:** 1279-1290.
125. Xie Z, Zhang ZL, Zou X, Huang J, Ruas P, Thompson D, Shen QJ (2005) Annotations and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiol **137:** 176-189.
126. Yin LN, Mano JC, Wang SW, Tsuji W, Tanaka K (2010) The involvement of lipid peroxide-derived aldehydes in aluminium toxicity of tobacco roots. Plant Physiol **152:** 1406-1417.
127. Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JD, Doke N (2003) *Nicotiana benthamiana* gp91phox homologs NbrbohA and NbrbohB participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell **15:** 706-718.

128. Yuasa T, Ichimura K, Mizoguchi T, Shinozaki K (2001) Oxidative stress activates ATMPK6, an *Arabidopsis* homologue of MAP kinase. Plant Cell Physiol **42:** 1012-1016.
129. Zhang J, Addepalli B, Yun KY, Hunt AG, Xu R, Rao S, Li QQ, Falcone DL (2008) A polyadenylation factor subunit implicated in regulating oxidative signaling in *Arabidopsis thaliana*. PLoS One **3:** e2410.
130. Zhang A, Zhang J, Ye NH, Cao JM, Tan MP, Zhang JH, Jiang MY (2010) ZmMPK5 is required for the NADPH oxidase-mediated self-propagation of apoplastic H_2O_2 in brassinosteroid-induced antioxidant defense in leaves of maize. J Exp Bot **61:** 4399-4411.
131. Zimmermann P, Heinlein C, Orendi G, Zentgraf U (2006) Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. Plant Cell Environ **29:** 1049-1060.
132. Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JDG, Felix G Boller T (2004) Bacterial disease resistance in *Arabidopsis* through flagellin perception. Nature **428:** 764-767.

Published in Biochem Biophys Res Commun (2008) 375: 639-644.

Chapter 2

Arabidopsis AAL-toxin-resistant mutant *atr1* shows enhanced tolerance to programmed cell death induced by reactive oxygen species

Tsanko S. Gechev[1,2*]
Margriet A. Ferwerda[2]
Nikolay Mehterov[1]
Christophe Laloi[3]
Muhammad Kamran Qureshi[2]
Jacques Hille[2]

[1] Department of Plant Physiology and Plant Molecular Biology, University of Plovdiv, 24 Tsar Assen str., Plovdiv 4000, Bulgaria

[2] Department Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

[3] Institute of Plant Sciences, ETH Zurich, Universitätsstrasse 2, CH-8092 Zurich, Switzerland

Abstract

The fungal AAL-toxin triggers programmed cell death (PCD) through perturbations of sphingolipid metabolism in AAL-toxin-sensitive plants. While *Arabidopsis* is relatively insensitive to the toxin, the *loh2* mutant exhibits increased susceptibility to AAL-toxin due to the knockout of a gene involved in sphingolipid metabolism. Genetic screening of mutagenized *loh2* seeds resulted in the isolation of AAL-toxin-resistant mutant *atr1*. *atr1* displays a wild-type phenotype when grown on soil but it develops less biomass than *loh2* on media supplemented with 2% and 3% sucrose. *atr1* was also more tolerant to the reactive oxygen species-generating herbicides aminotriazole (AT) and paraquat. Microarray analyses of *atr1* and *loh2* under AT-treatment conditions that trigger cell death in *loh2* and no visible damage in *atr1* revealed genes specifically regulated in *atr1* or *loh2*. In addition, most of the genes strongly downregulated in both mutants were related to cell wall extension and cell growth, consistent with the apparent and similar AT-induced cessation of growth in both mutants. This indicates that two different pathways, a first controlling growth inhibition and a second triggering cell death, are associated with AT-induced oxidative stress.

Key words: AAL-toxin; hydrogen peroxide; reactive oxygen species; sphingolipid metabolism; programmed cell death

1. Introduction

Programmed cell death (PCD) is essential for a number of plant developmental processes and responses to pathogens (Gechev and Hille, 2005). Examples of developmentally regulated processes in which PCD is involved include embryo development, nucellar degeneration, maturation of tracheal elements and epidermal trichomes, formation of lace leaf shape, and leaf senescence (Gechev et al., 2006). Another type of PCD is represented by the hypersensitive response, a defense reaction in which plant cells in and around the site of pathogen infection die in order to physically restrict the spread of the pathogen (Dangl and Jones, 2001). While in the above examples cell death is beneficial and/or essential for plant development and survival, some necrotrophic pathogens can secrete toxins that cause cell death in healthy tissues so that the pathogens can feed on the dead tissues (Gechev et al., 2004).

The fungal AAL-toxin triggers cell death through perturbations of sphingolipid metabolism in AAL-toxin-sensitive tomato (Spassieva et al., 2002). The toxin inhibits ceramide synthase, a key enzyme in sphingolipid synthesis, which leads to accumulation of precursors and depletion of complex sphingolipids. Tomato plants sensitive to the AAL-toxin have a mutation in the *Asc* gene that is most likely a component of the ceramide synthase (Brandwagt et al., 2000). The *Arabidopsis thaliana loh2* mutant is more sensitive to the AAL-toxin than the wild type due to the knockout of a gene homologous to the tomato *Asc* gene (Gechev et al., 2004). Microarray analyses of AAL-toxin-induced cell death in *loh2* revealed induction of hydrogen peroxide-responsive genes and genes that are involved in the oxidative burst at early time points preceding visible cell death symptoms (Gechev et al., 2004). This indication of oxidative burst in AAL-toxin-treated plants was in agreement with previous studies demonstrating accumulation of reactive oxygen species in *Arabidopsis* plants treated with fumonisin B_1 (FB_1), an AAL-toxin analogue (Asai et al., 2000). Moreover, a recently identified FB_1 resistant mutant compromised in serine palmitoyl transferase, a key enzyme of *de novo* sphingolipid synthesis, failed to generate ROS and to initiate cell death upon FB_1 treatment (Shi et al., 2007).

This paper describes a genetic approach carried out to isolate a mutant called *atr1* (AAL-toxin-resistant1) that survives AAL-toxin treatment, and its characterization in respect to reactive oxygen species-induced cell death. Microarray experiments of *atr1* and *loh2* under conditions that induce cell death only in *loh2* followed by bioinformatics analysis were carried out in order to identify genes with a potential role in the cell death process.

2. Materials and Methods

2.1. Plant material, isolation of mutants, growth conditions, stress treatments and cell death assessment

Fourty thousand seeds from *Arabidopsis thaliana loh2* mutant, described earlier (Gechev et al., 2004), were mutagenized with 0.1–0.3% ethane methyl sulfonate for eight hours. After extensive washing, the mutagenized seeds were planted on soil in pools and grown under standard greenhouse conditions (14 h light/10 h dark period, photosynthetic photon flux density 400 µmol. m^{-2}. s^{-1}, 22°C and relative humidity 70%). Screening for resistance to AAL-toxin was done by plating the self-pollinated progeny seeds from M1 plants on growth media containing 40 nM of AAL-toxin and grown in a climate room under the following conditions: 60 µmol. m^{-2}. s^{-1}, 22°C. AAL-toxin-resistant survivors, including *atr1*, were transferred to the greenhouse and seeds collected for further analysis. Light stress responses of *loh2* and *atr1* were evaluated by shifting in vitro-grown plants from 60 to 600 µmol. m^{-2}. s^{-1}. Chilling stress was applied by shifting one-week grown plants from 60 µmol. m^{-2}. s^{-1}, 22°C to 240 µmol. m^{-2}. s^{-1}, 4°C for 1, 2 and 3 days. Salt stress was applied by growing plants on media supplemented with 60, 80 or 100 mM NaCl. Abiotic stress tolerance was evaluated by measuring variable fluorescence Fv/Fm with Fluorcam 700 MF, fresh weight, and chlorophyll content. Assessment for tolerance to ROS-induced programmed cell death was done by plating *atr1* and *loh2* seeds on media containing either 7 µM aminotriazole (AT) or 0.5 µM paraquat and measuring the relative loss of fresh weight, chlorophyll, and

visible cell death. Chlorophyll content was measured photometrically as previously described (Gechev et al., 2003).

2.2. Isolation of RNA and microarray experiments

Samples for RNA isolation and microarray analysis were collected from *atr1* and *loh2* mutants grown on media with or without 7 µM AT four days after germination. One week after germination, this concentration of AT lead to mortality in *loh2* and no death symptoms in *atr1*. RNA was isolated using RNA Plant Mini Kit (Qiagen) as previously described (Gechev et al., 2005). Microarray experiments with two biological repetitions were performed in compliance with the MIAME standards (Brazma et al., 2001). The *Arabidopsis2* oligonucleotide array of Agilent Technologies was used, representing 21 500 genes. The labeling, hybridization, and data extraction were done at ServiceXS (The Netherlands) according to the instructions of Agilent Technologies as previously described (Gechev et al., 2004).

2.3. Bioinformatics analysis

Datasets resulting from the microarray experiments were subjected to hierarchical complete linkage clustering using the Cluster/Treeview program (Gadjev et al., 2006). Transcripts showing a minimum five-fold difference in expression in at least one experiment were clustered in two dimensions: transcripts and mutants. Promoter regions of genes from clusters with similar mode of expression and known cis-regulatory elements of the genes were retrieved from the *Arabidopsis cis*-regulatory elements database, Ohio State University (http://arabidopsis.med.ohio-state.edu/). Search for new common cis-regulatory elements was done with the MEME/MAST system developed at Purdue University (http://meme.nbcr.net/meme/intro.html).

3. Results

3.1. Isolation of *atr1*

The *loh2* mutant of *Arabidopsis* is sensitive to AAL-toxin due to knockout of a gene involved in sphingolipid metabolism (Gechev et al., 2004). Fourty thousand seeds from *loh2* were chemically mutagenized with ethane methyl sulfonate, germinated on soil, self-pollinated and the resulting progeny plated on AAL-toxin-containing media in order to isolate mutants that are more tolerant to AAL-toxin than the original *loh2* background. While the wild type *Arabidopsis* is resistant to 200 nM AAL-toxin, the *loh2* mutant develops cell death symptoms at 20 nM AAL-toxin already and 40 nM of the toxin leads to lethality. Thirty independent survivors were isolated using a concentration of 40 nM AAL-toxin as a screening threshold. The first one of them, named *atr1* (AAL-toxin resistant1), was selected for further analysis (figure 1). Genetic studies by crossing *atr1* with the wild type and studying the progeny indicated that *atr1* was recessive (data not shown). While *atr1* displays a wild-type phenotype when grown on soil, it develops less biomass than *loh2* on growth media supplemented with 2% and 3% sucrose (table 1).

3.2. *atr1* is more tolerant to ROS-generating herbicides

Earlier studies indicated that the AAL-toxin causes induction of ROS-associated genes and H_2O_2 accumulation that precedes the cell death (Gechev et al., 2004). To investigate this relation, the *atr1* was also tested for tolerance to PCD induced by reactive oxygen species (figure 2). The catalase inhibitor aminotriazole (AT) leads to H_2O_2 accumulation and subsequent cell death (Gechev et al., 2002), whereas paraquat causes superoxide-dependent cell death (Vranova et al., 2002). Application of either AT or paraquat in plant growth media caused reduction in growth as measured by fresh weight loss (figure 2), reduction in total chlorophyll content (figure 2) and eventually death of *loh2*. However, *atr1* was more tolerant to both paraquat and AT than *loh2*, as estimated by the lack of cell death, smaller decrease in fresh weight and more chlorophyll. *atr1* was asymptomous on 7 µM AT while the original *loh2* background died (figure 3). AT inhibits catalase activity in

Figure 1: Screening of *atr1* on media with AAL-toxin. M2 seeds from ethane methyl sulfonate- mutagenized AAL-toxin-sensitive *loh2* plants were plated on plant growth media supplemented with 40 nM AAL-toxin. The picture shows the survivor *atr1* one week after germination.

Table 1: Fresh weight of 10 day-old *loh2* and *atr1* seedlings grown on plant growth media supplemented with different concentrations of sucrose.

	Sucrose concentration (%)			
	0	**1**	**2**	**3**
loh2	1.73 ± 0.43	3.26 ± 0.15	8.33 ± 0.44	8.1 ± 0.38
atr1	1.68 ± 0.09	3.17 ± 0.44	3.2 ± 0.43	3.83 ± 0.17

The fresh weight (mg) of 40 seedlings was determined for each genotype in each condition. Data represent the mean of one seedling ± SD obtained from three independent experiments.

both *atr1* and *loh2* plants with the same efficiency (data not shown), suggesting that *atr1* may act downstream of hydrogen peroxide accumulation. As both AAL-toxin- and AT-induced cell deaths are light-dependent processes, we investigated the responses of *loh2* and *atr1* plants to light stress. Altering the light intensity from 60 to 600 μmol. m^{-2}. s^{-1} resulted in similar light stress responses in both mutants (data not shown). *atr1* and *loh2* were also similar in their responses to chilling and salt stress (data not shown).

3.3. Gene expression analyses in *loh2* and *atr1* plants exposed to AT

While *loh2* plants on media supplemented with AAL-toxin or paraquat die at a very early stage without developing fully expanded cotyledons, AT treatment allow *loh2* plants to develop well-expanded cotyledons before they start dying

and therefore constitute a very suitable system to analyze gene expression under cell death inducing conditions (figure 3). Microarray analyses of *loh2* and *atr1* under conditions that trigger cell death in *loh2* and no visible damage in *atr1* were carried out to identify genes specifically regulated in the two mutants. Both mutants were plated on medium without or with 7 μM AT, a condition that is eventually lethal for *loh2* and asymptomous for *atr1* (figure 3). Samples for microarray analysis were collected on the fourth day after germination, two days before the first visible cell death symptoms in *loh2*. The complete datasets are available as supplementary material. Genes (219) with an at least 5-fold increase or decrease in AT-treated *loh2* or *atr1* plants compared to untreated plants were subjected to hierarchical complete linkage clustering analysis (Gadjev et al., 2006) and the results presented in figure 4. The most regulated genes from figure 4 are presented in table 2. The clustering revealed four prominent clusters: genes upregulated in both *loh2* and *atr1* (cluster A), genes upregulated in *loh2* and downregulated or not regulated in *atr1* (B), genes downregulated or not regulated in *loh2* and upregulated in *atr1* (C), and genes downregulated in both mutants (D). The two biggest clusters in figure 4, namely A and D, consist of genes co-regulated in both mutants, either coinduced (A) or corepressed (D). The cluster A comprise nitrate and ammonium transporters, peroxidases, transcription factors, a transposase, HSC70, WRKY and NAM family transcription factors, and a number of genes with unknown function. Twenty genes were exclusively induced in *loh2* and not induced or downregulated in *atr1* (cluster B), including four heat shock genes, two glycosyl transferases, a peptidylprolyl isomerase, and seven genes encoding for proteins with unknown functions. Seventeen genes were induced only in *atr1*, including two nicotianamine synthases, an allergen, and five genes with unknown functions. The cluster D of genes downregulated in both mutants contain two trypsin inhibitors, a protein kinase, arabinogalactans, expansins, xyloglucan endotransglycosylases, a pectinesterase and proline-rich proteins.

In order to find common and specific cis-regulatory elements in the promoter regions of coregulated genes, promoters of those genes were analyzed for presence of known elements. Sequence binding sites for transcription factors of the WRKY,

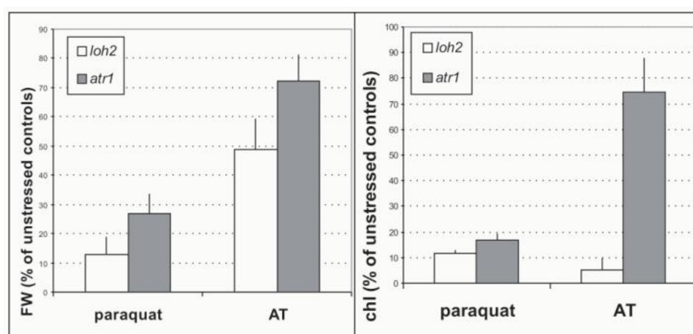


Figure 2: *atr1* is more tolerant to reactive oxygen species-induced cell death. Seeds from *loh2* and *atr1* mutants were plated on plant growth media supplemented either with 0.5 µM paraquat or with 7 µM AT in order to assess their tolerance to cell death induced by superoxide radicals or hydrogen peroxide, respectively. Data represents the loss of fresh weight (FW) or chlorophyll (chl) of *loh2* and *atr1* on media supplemented with paraquat or aminotriazole and compared with *loh2* and *atr1* grown without paraquat and aminotriazole (controls). Samples for the measurements were collected one week after germination. Data are means of three independent biological experiments ± SD.

MYB, bZIP, GATA zinc finger family were frequent in many of the regulated genes, although none of those were present in all of the regulated genes. Some of the AT-regulated genes contained one or several cis-regulatory elements reported to be involved in H_2O_2 signaling, including the recently identified B-box and NRXe-2 elements (Geisler et al., 2008; Ho et al., 2008). A computational approach could not detect new common cis-elements specific for the gene clusters.

4. Discussion

Taking advantage of a system for studying cell death triggered by AAL-toxin, second-site mutants more tolerant to AAL-toxin than the initial toxin-sensitive *loh2* background have been isolated. Earlier investigations revealed that AAL-toxin-induced cell death is connected with a burst of H_2O_2 and the activation of H_2O_2-responsive/generating genes (Gechev et al., 2004). Moreover, comparative transcriptome analyses of ROS-related experiments showed that the responses to

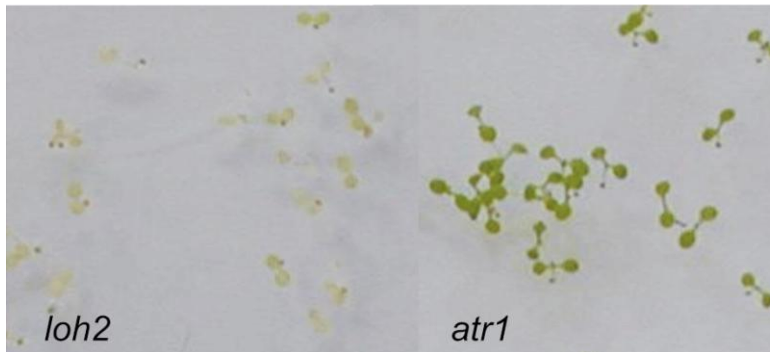


Figure 3: *atr1* is more tolerant to cell death induced by the hydrogen peroxide-generating catalase inhibitor aminotriazole. Seeds from the AAL-toxin-sensitive *loh2* and AAL-toxin-resistant *atr1* mutants were germinated on media supplemented with 7 µM aminotriazole. The picture is taken ten days after germination. On the left, *loh2*; on the right, *atr1*. Under these conditions, all *loh2* plants died, while no visible cell death symptoms were observed in *atr1*.

AAL-toxin and AT treatments fall into a common gene cluster of photorespiratory H_2O_2 (Gadjev et al., 2006). The isolation of *atr1* with enhanced tolerance to both AAL-toxin and ROS-generating agents is an additional genetic evidence for the link between AAL-toxin- and ROS-induced cell death. However, there is a clear difference between the symptoms of AAL-toxin-, AT- and paraquat-induced cell death, suggesting that despite common production of H_2O_2 and similarities in gene expression, these treatments may activate different cell death signaling pathways. Recent discoveries of promoter regions and cis-regulatory elements specific for distinct types of ROS further support the notion for different ROS signaling pathways (Ho et al., 2008; Shao et al., 2007).

Both AAL-toxin- and AT-induced PCD are light-dependent processes. *loh2* and *atr1* showed similar responses towards light stress, suggesting that the mutation is specific to cell death and not to light-dependent stress responses. The two mutants are also undistinguishable in their responses to other abiotic stresses tested. In contrast, *atr1* grows slower than *loh2* on growth media supplemented with 2–3% sucrose but not on media without or with 1% sucrose and on soil (table 1). The

Table 2: Genes most altered in expression during AT-induced cell death.

Gene	TAIR locus	*loh2*	*atr1*
high-affinity nitrate transporter	At1G08090	8.205	57.225
peroxidase	At2G18150	5.425	8.86
PEP carboxylase	At3G42628	5.685	6.14
transposase	At1G42110	7.445	5.755
HSC70	At5G02490	5.59	3.395
isocytrate lyase	At3G21720	9.77	2.67
hsp17	At3G46230	12.735	1.425
hsp17.6	At5G12030	8.625	1.3
peptidylprolyl isomerase	At5G48570	3.63	1.04
allergen-like	At4G17030	1.61	4.72
nicotianamine synthase	At5G56080	2.02	4.79
xyloglucan endotransglycosylase	At4G28850	-32.34	-56.05
xyloglucan endotransglycosylase	At2G18800	-30.595	-15.865
extensin-like	At5G46890	-28.08	-23.735
proline-rich protein	At2G33790	-27.72	-30.665
extA	At5G46900	-21.76	-20.9
arabinogalactan AGP13	At4G26320	-10.43	-7.795
cytochrome p450	At2G25160	-10.145	-4.23
pectinesterase family	At5G04960	-8.41	-54.255
putative protein	At5G62340	-7.985	-14.92
hypothetical protein	At1G09720	-6.72	-7.175
putative protein	At4G25250	-7.35	-6.785
trypsin inhibitor	At3G04320	-6.29	-7.585
hypotetical	At3G18295	-12.57	-1.26
ripening-related	At5G51520	-1.08	-8.18

Arabidopsis thaliana loh2 and *atr1* mutants were grown on media without or with 7 μM AT and samples collected two days before cell death symptoms in *loh2*. Data are means of two biological replicates. Positive values indicate upregulated genes while negative values indicate downregulated genes.

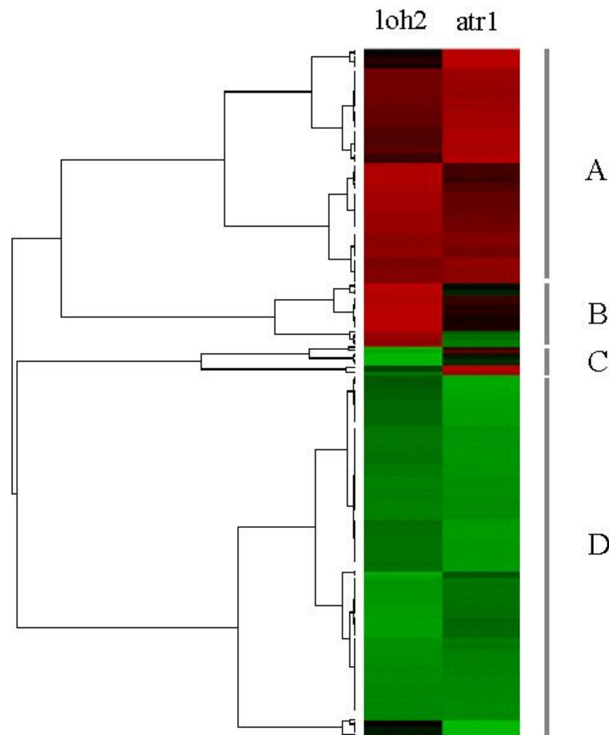


Figure 4: Hierarchical complete linkage clustering of genes most altered in expression during AT-induced cell death. Samples from *Arabidopsis loh2* and *atr1* mutants grown on media without and with AT were collected on the fourth day after germination, two days before the cell death symptoms in *loh2*, and microarray analysis performed as described in materials and methods. The figure presents hierarchical average linkage clustering of 219 genes up- or down-regulated at least 5-fold by AT treatment in either *loh2* (first column) or *atr1* (second column), average from two biological replicates. Each row represents the expression profile of an individual gene. Red color indicates up-regulation while green indicates down-regulation. The color intensity corresponds to the extent of induction/repression. A, B, C and D on the right are the four main gene clusters.

reason for this phenomenon is unknown but it could be a 'trade-off' price for the cell death tolerance of *atr1*.

AAL-toxin-induced PCD is connected with both depletion of complex ceramides and accumulation of precursors, as inhibiting serine palmitoyl transferase

abolishes the cell death (Spassieva et al., 2002). Likewise, mutation of serine palmitoyl transferase in *fbr11* results in the absence of ROS burst upon FB_1-treatment and the lack of cell death (Asai et al., 2000). Thus, *atr1* seems to be different from *fbr11*. The inhibition of catalase activity by aminotriazole in both *loh2* and *atr1* suggests that the *atr1* mutation may be interfering with signal perception or/and transduction rather than hydrogen peroxide accumulation. Alternatively, the mutation may inactivate a gene essential for regulation or execution of the cell death program that is situated below the hydrogen peroxide perception and transduction.

Previous studies of the transcriptome during hydrogen peroxide-mediated cell death in wild type plants revealed similarities in gene expression with our datasets, for example induction of hydrogen peroxide-sensitive HSC70, transcription factors and peroxidases (Desikan et al., 2001; Gechev et al., 2005; Vandenabeele et al., 2004; Zago et al., 2006). The results here reveal new H_2O_2-regulated genes, including a nitrate transporter, trypsin inhibitors, and protein kinases. Microarray analyses also revealed that *loh2* and *atr1* have very similar patterns of gene expression and only a small percentage of the transcripts are exclusively regulated in *loh2* or in *atr1*. Among them, two heat shock protein genes and a peptidylprolyl isomerase are induced only in *loh2*, an allergen-like gene is induced only in *atr1*, and a ripening-related gene is exclusively repressed in *atr1*. Heat shock proteins have diverse functions in plant biology and are rapidly induced under various conditions, including heat shock and oxidative stress (Gechev et al., 2005; Lin et al., 2001; Vandenabeele et al., 2004). While heat shock genes are extensively studied in plants, there is little functional data on plant peptidylprolyl isomerases (Romano et al., 2005).

Most of the repressed genes in *loh2* and *atr1* were related to cell wall metabolism, which in turn can govern cell growth and development. Expansins are primary wall-loosening factors that induce turgor-driven wall extension, while xyloglucan endotransglucosylases as secondary factors reform the xyloglucan-cellulose wall structure, rendering it more responsive to the primary wall-loosening events (Li et al., 2003). The loose structure of the cell wall then allows growth of the

cell. Repression of those genes, therefore, may inhibit plant growth and development. Indeed, growth inhibition that precedes cell death is clearly observed in *loh2* and to a lesser extent in *atr1*. However, the growth inhibition in *atr1* is eventually overcome and the plants continue to develop. In addition to extensins and xyloglucan endotransglucosylases, the arabinogalactan proteins have also been implicated as regulators of cell growth and mediators of cell-cell interactions (Pilling and Hofte, 2003). Downregulation of these genes is consistent with cessation of growth in both mutants and indicates that two distinct pathways may be activated by AT-induced oxidative stress: one controlling growth inhibition, active in both *loh2* and *atr1*, and another triggering cell death, executed in *loh2* but abolished in *atr1*.

Acknowledgements

Authors wish to thank K. Apel and I. Gadjev for helpful discussions. This work was supported in part by grants from the IAEA (contract 13220), the Swiss NSF (SCOPES program) and the NSF of Bulgaria (projects G5/UB3).

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version of Biochem Biophys Res Commun (2008) 375: 639-644.

References

1. Asai T, Stone JM, Heard JE, Kovtun Y, Yorgey P, Sheen J, Ausubel FM (2000) Fumonisin B1-induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. Plant Cell **12:** 1823-1835.
2. Brandwagt BF, Mesbah LA, Takken FLW, Laurent PL, Kneppers TJA, Hille J, Nijkamp HJJ (2000) A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp *lycopersici* toxins and fumonisin B-1, Proc Natl Acad Sci USA **97:** 4961-4966.
3. Brazma A, Hingamp P, Quackenbush J, Sherlock G, Spellman P, Stoeckert C, Aach J, Ansorge W, Ball CA, Causton HC, Gaasterland T, Glenisson P, Holstege FCP, Kim IF, Markowitz V, Matese JC, Parkinson H, Robinson A, Sarkans U,

Schulze-Kremer S, Stewart J, Taylor R, Vilo J, Vingron M (2001) Minimum information about a microarray experiment (MIAME) - toward standards for microarray data. Nature Genet **29:** 365-371.

4. Dangl JL, Jones JD (2001) Plant pathogens and integrated defence responses to infection. Nature **411:** 826-833.
5. Desikan R, Mackerness SAH, Hancock JT, Neill SJ (2001) Regulation of the *Arabidopsis* transcriptome by oxidative stress. Plant Phys **127:** 159-172.
6. Gadjev I, Vanderauwera S, Gechev T, Laloi C, Minkov I, Shulaev V, Apel K, Inzé D, Mittler R, Van Breusegem F (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in *Arabidopsis*. Plant Phys **141:** 434-445.
7. Gechev T, Gadjev I, Van Breusegem F, Inzé D, Dukiandjiev S, Toneva V, Minkov I (2002) Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. Cell Mol Life Sci **59:** 708-714.
8. Gechev T, Willekens H, Van Montagu M, Inzé D, Van Camp W, Toneva V, Minkov I (2003) Different responses of tobacco antioxidant enzymes to light and chilling stress. J Plant Phys **160:** 509-515.
9. Gechev TS, Gadjev IZ, Hille J (2004) An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity of programmed cell death in plants. Cell Mol Life Sci **61:** 1185-1197.
10. Gechev TS. Hille J (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol **168:** 17-20.
11. Gechev TS, Minkov IN, Hille J (2005) Hydrogen peroxide-induced cell death in *Arabidopsis*: Transcriptional and mutant analysis reveals a role of an oxoglutarate-dependent dioxygenase gene in the cell death process. IUBMB Life **57:** 181-188.
12. Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays **28:** 1091-1101.
13. Geisler M, Kleczkowski L, Karpinski S (2008) A universal algorithm for genome-wide in silicio identification of biologically significant gene promoter putative cis-regulatory-elements; identification of new elements for reactive oxygen species and sucrose signaling in *Arabidopsis*. Plant J **45:** 384-398.
14. Ho LH, Giraud E, Uggalla V, Lister R, Clifton R, Glen A, Thirkettle-Watts D, Van Aken O, Whelan J (2008) Identification of regulatory pathways controlling gene expression of stress-responsive mitochondrial proteins in *Arabidopsis*. Plant Phys **147:** 1858-1873.
15. Li Y, Jones L, McQueen-Mason S (2003) Expansins and cell growth. Curr Opin Plant Biol **6:** 603-610.

16. Lin BL, Wang JS, Liu HC, Chen RW, Meyer Y, Barakat A, Delseny M (2001) Genomic analysis of the Hsp70 superfamily in *Arabidopsis thaliana*. Cell Stress Chap **6:** 201-208.
17. Pilling E, Hofte H (2003) Feedback from the wall. Curr Opin Plant Biol **6:** 611-616.
18. Romano P, Gray J, Horton P, Luan S (2005) Plant immunophilins: functional versatility beyond protein maturation. New Phytol **166:** 753-769.
19. Shao N, Krieger-Liszkay A, Schroda M, Beck CF (2007) A reporter system for the individual detection of hydrogen peroxide and singlet oxygen: its use for the assay of reactive oxygen species produced in vivo. Plant J **50:** 475-487.
20. Shi LH, Bielawski J, Mu JY, Dong HL, Teng C, Zhang J, Yang XH, Tomishige N, Hanada K, Hannun YA, Zuo JR (2007) Involvement of sphingoid bases in mediating reactive oxygen intermediate production and programmed cell death in *Arabidopsis*. Cell Res **17:** 1030-1040.
21. Spassieva SD, Markham JE, Hille J (2002) The plant disease resistance gene Asc-1 prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death. Plant J **32:** 561-572.
22. Vandenabeele S, Vanderauwera S, Vuylsteke M, Rombauts S, Langebartels C, Seidlitz HK, Zabeau M, Van Montagu M, Inzé D, Van Breusegem F (2004) Catalase deficiency drastically affects gene expression induced by high light in *Arabidopsis thaliana*. Plant J **39:** 45-58.
23. Vranova E, Atichartpongkul S, Villarroel R, Van Montagu M, Inzé D, Van Camp W (2002) Comprehensive analysis of gene expression in *Nicotiana tabacum* leaves acclimated to oxidative stress. Proc Natl Acad Sci USA **99:** 10870-10875.
24. Zago E, Morsa S, Dat J, Alard P, Ferrarini A, Inzé D, Delledonne M (2006) Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. Plant Phys **141:** 401-411.

Published in Acta Physiol Plant (2011) 33: 375-382.

Chapter 3

Isolation and characterization of *Arabidopsis* mutants with enhanced tolerance to oxidative stress

Muhammad Kamran Qureshi[1,3]

Vesela Radeva[2,3]

Todor Genkov[2]

Ivan Minkov[2]

Jacques Hille[1]

Tsanko S. Gechev[2]

[1] Department Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

[2] Department of Plant Physiology and Plant Molecular Biology, University of Plovdiv, 24 Tsar Assen str., Plovdiv 4000, Bulgaria

[3] these authors contributed equally to this work

Abstract

We have previously reported a method for isolation of mutants with enhanced tolerance to the fungal AAL toxin and given a detailed characterization of *atr1* (AAL toxin resistant, Gechev et al. in Biochem Biophys Res Commun 375: 639-644, 2008). Herewith, we report eight more mutants with enhanced tolerance to the AAL toxin. Phenotypic analysis showed that six of the mutants were reduced in size compared with their original background *loh2*. Furthermore, *atr2* showed delayed flowering and senescence. The mutants were also evaluated for oxidative stress tolerance by growing them on ROS-inducing media supplemented with either aminotriazole or paraquat, generating, respectively, H_2O_2 or superoxide radicals. Oxidative stress, confirmed by induction of the marker genes, *HIGH AFFINITY NITRATE TRANSPORTER* At1g08090 and *HEAT SHOCK PROTEIN 17* At3g46230, inhibited growth of all lines. However, while the original background *loh2* developed necrotic lesions and died rapidly on ROS-inducing plant growth media, *atr1*, *atr2*, *atr7* and *atr9* remained green and viable. The tolerance against oxidative stress-induced cell death was confirmed by fresh weight and chlorophyll measurements. Real-time PCR analysis revealed that the expression of the *EXTENSIN* gene At5g46890, previously shown to be downregulated by aminotriazole in *atr1*, was repressed in all lines, consistent with the growth inhibition induced by oxidative stress. Taken together, the data indicate a complex link between growth, development and oxidative stress tolerance and indicates that growth inhibition can be uncoupled from oxidative stress-induced cell death.

Key words: AAL-toxin, programmed cell death, aminotriazole, paraquat, hydrogen peroxide, oxidative stress

1. Introduction

Oxidative stress-induced programmed cell death (PCD) can occur under many unfavorable environmental conditions as well as in biotic interactions (Apel and Hirt, 2004). Furthermore, reactive oxygen species (ROS)-induced cell death is also observed during several developmental processes (Gadjev et al., 2008). Oxidative stress-induced PCD is a genetically controlled process triggered mostly by hydrogen peroxide (H_2O_2) and also by other types of ROS, including superoxide radicals and singlet oxygen (Gadjev et al., 2008; Gechev et al., 2006; Gechev and Hille, 2005).

In addition to the abiotic stress factors mentioned earlier, elevated levels of H_2O_2 and subsequently H_2O_2-induced cell death can be triggered by catalase deficiency, especially under conditions that promote photorespiration such as high light intensity (Gechev et al., 2005; Vanderauwera et al., 2005). Catalase deficiency can be induced by either silencing the catalase gene(s) or inhibiting catalase activity by the catalase inhibitor aminotriazole (AT) (Gechev et al., 2005; Vanderauwera et al., 2005). Moreover, AT can be used as a screening agent for identifying mutants more tolerant to oxidative stress (Gechev et al., 2008).

The fungal AAL toxin causes PCD through perturbations in sphingolipid metabolism (Brandwagt et al., 2000; Spassieva et al., 2002). The toxin inhibits ceramide synthase, a key enzyme in sphingolipid biosynthesis, which leads to accumulation of precursors and depletion of complex sphingolipids. In tomato, the sensitivity to AAL toxin is conferred by a mutation in the *Asc* gene that is most likely a component of the ceramide synthase (Brandwagt et al., 2000). Likewise, the *loh2* mutant, knockout of the *Asc* homologous gene in *Arabidopsis thaliana*, has increased sensitivity to AAL toxin (Gechev et al., 2004). The AAL toxin-induced PCD in *Arabidopsis* is associated with elevated levels of H_2O_2 that precede cell death (Gechev et al., 2004). The burst of ROS was confirmed by microarray analyses of AAL toxin induced cell death in *loh2*. This analysis revealed induction of H_2O_2-responsive genes and genes that are involved in the oxidative burst at early time points preceding visible cell death symptoms (Gechev et al., 2004). The oxidative burst in AAL toxin-treated plants was in agreement with previous studies

demonstrating accumulation of ROS in *Arabidopsis* plants treated with fumonisin B_1 (FB_1), an AAL toxin analog, and by the recently identified FB_1 resistant mutant compromised in serine palmitoyl transferase, a key enzyme of *de novo* sphingolipid synthesis. This mutant failed to generate ROS and to initiate cell death upon FB_1 treatment (Asai et al., 2000; Shi et al., 2007). We have previously reported a new method for isolation of mutants with enhanced tolerance to the fungal AAL toxin as well as a detailed characterization of one such mutant, named *atr1* (AAL toxin resistant, Gechev et al., 2008). In this paper, we report eight more mutants with enhanced tolerance to the AAL toxin. The new mutants are phenotyped and their tolerance toward ROS-induced cell death evaluated. In addition, we discuss the link between oxidative stress and plant development.

2. Materials and Methods

2.1. Plant growth conditions, mutagenesis, and mutant screening

Plants were grown in a greenhouse under standard conditions (14 h light/10 h dark period, photosynthetic photon flux density 400 µmol. m^{-2}. S^{-1}, 22°C and relative humidity 70%) or in a climate room (14 h light/10 h dark period, photosynthetic photon flux density 100 µmol. m^{-2}. s^{-1}, 22°C and relative humidity 70%). Seeds from the AAL-toxin-sensitive *A. thaliana loh2* mutant were mutagenized with 0.1, 0.2 and 0.3% ethane methyl sulfonate for 8 h, washed extensively and planted on soil in the greenhouse to self-pollinate and the progeny collected (M2). Isolation of AAL toxin-resistant *atr* mutants was done by plating seeds from M2 plants on petri dishes with Murashige and Skoog (MS) media containing 40 nM of AAL toxin and grown in a climate room. The independent AAL toxin-resistant survivors from different pools were transferred to the greenhouse and seeds collected for further analysis.

2.2. Stress treatments and evaluation of stress tolerance

Oxidative stress was applied by plating the seeds on media containing either AT or paraquat at concentrations of 5, 7, or 9 µM for AT and 0.5, 1 or 1.5 µM for

paraquat. Plants were grown for 2 weeks; fresh weight and chlorophyll content were assessed 7 and 10 days after germination. Chlorophyll content was measured photometrically as previously described (Gechev et al., 2002; Gechev et al., 2003). Briefly, the pigments were extracted with 80% acetone at 4°C overnight, samples centrifuged to remove solid particles, and absorption of chlorophyll *a* and *b* measured at 663 and 647 nm. Chlorophyll content was calculated as microgram per milligram fresh weight.

2.3. Isolation of RNA and real-time PCR measurements

Total RNA was isolated using TRIZOL® reagent (Invitrogen), following the manufacturer's guidelines. RNA was extracted from 4 days old seedlings planted on MS media with or without 7 µM AT. RNA was quantified at 260 nm and its quality was checked on gel. The following genes and corresponding primer pairs were used in the real-time PCR analysis: *HIGH AFFINITY NITRATE TRANSPORTER* (At1g08090), primer pairs CCATGGGAGTTGAGTTGAGC and AAAGTCAGAT GCGTAGCCTCC; *HSP17* (At3g46230), GTATGGGATCCGTTCGAAGG and TC TTCCTTCTTAAGCCCAGGC; *EXTENSIN* (At5g46890), CAAGAGCTACCACA AGAAGCC and GAGCGCAACAGTTGGACG; *PROFILIN 1* (At2g19760), AGAG CGCCAAATTTCCTCAG and CCTCCAGGTCCCTTCTTCC. Primer pairs were designed to cross exon-intron boundaries to minimize genomic DNA amplification. Reverse transcription products were obtained by using the RevertAid™ First strand cDNA Synthesis Kit (Fermentas) according to manufacturer's instructions. Real-time PCR was performed with 7500 Real-Time PCR machine (Applied Biosystems). Reaction mixture per well contained: 12.5 ll Maxima™ SYBR Green/ROX qPCR Master Mix (2x) (Fermentas), 1 µl of each primer, 9.5 µl nuclease free water and 50 ng (1 µl) cDNA. *Profilin 1* was used as internal standard. The annealing temperature selected was 60°C and the program was run for 40 cycles. Melting curves did not show any unspecific products and primer dimers. Relative gene expression levels wereobtained using the ΔΔCt method (Winer et al., 1999). All samples were run in three independent repetitions.

2.4. Protein isolation and enzyme assays

Total protein was isolated and protein concentration quantified by the method of Bradford with a kit supplied by Bio-Rad as previously described (Gechev et al., 2002; Gechev et al., 2003). Catalase was determined photometrically following the decrease in absorbance of H_2O_2 at 240 nm and in gel assays (Gechev et al., 2002; Gechev et al., 2003).

3. Results and discussion

3.1. Isolation and phenotypic characterization of mutants more tolerant to cell death

The method for screening AAL toxin-tolerant mutants and isolation of *atr1* has previously been described in details (Gechev et al., 2008). Briefly, ethane methyl sulfonate-mutagenized seeds from the AAL toxin-sensitive line *loh2* plants were germinated on soil, self-pollinated and 40,000 batches of progeny screened on 40 mM AAL toxin-containing media, lethal to *loh2*. Nine *atr* mutants were isolated. Genetic studies by crossing *atr* with the wild type and studying the progeny indicated that *atr* mutants behaved as recessive mutants (data not shown). Wild-type plants are tolerant to as much as 200 nM AAL-toxin, which is more than tenfold higher than *loh2*, and can survive without any visible cell death symptoms. The tolerance of *atr* mutants to AAL toxin ranged between 40 and 100 nM. It is unclear why full resistance to the AAL toxin was never achieved. Sphingolipids are essential regulators not only of cell death, but also of development in both animal and plant cells (Chen et al., 2008; Teufel et al., 2009). This notion is supported by the various developmental defects in mutants with compromised very long-chain fatty acids that are components of sphingolipids and in mutants with impaired hydroxylation of long-chain bases (Bach et al., 2008; Chen et al., 2008; Zheng et al., 2005). It could be that all mutants, being in *loh2* background that lacks certain complex sphingolipids, are unable to proceed into mature plants when AAL toxin is present.

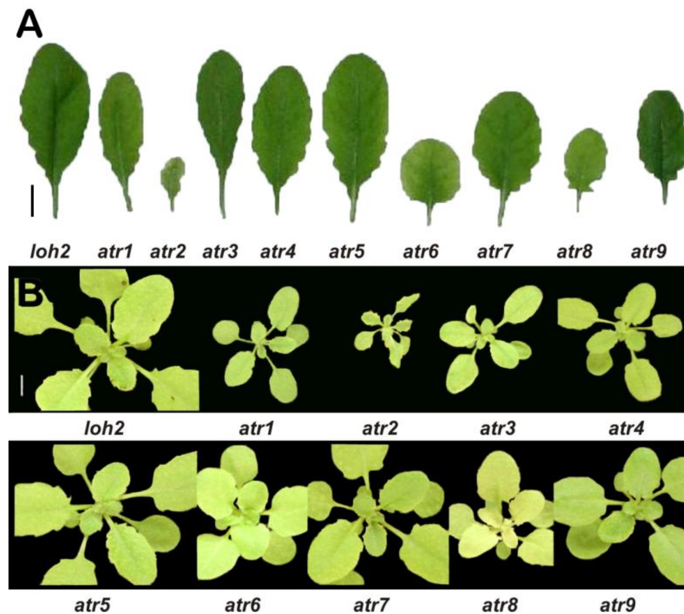


Figure 1: Phenotypes of *loh2* and *atr* mutants. *loh2* and *atr* mutants were grown on soil under standard greenhouse conditions (14 h light/10 h dark periods, PPFD 400 µmol m^{-2} s^{-1}, 22°C, and relative humidity 70%), and representative pictures taken at inflorescence emergence, 30 days after germination. A, Leaves of one-month-old plants; B, Phenotypes of different mutant lines. Bars represents 1 centimetre.

Some of the *atr* mutants were smaller in size than their original background *loh2* (figure 1). *loh2* has the same size as *A. thaliana* ecotype *Wassilewskija* or *A. thaliana* ecotype *Colombia* (data not shown). However, *atr1, atr2, atr6, atr7, atr8,* and *atr9* had smaller leaves and were reduced in size to different extents when grown on soil (figure 1). Furthermore, some of the mutants had altered leaf shape (figure 1). *atr6* had smaller adult leaves rounded in shape; remained dwarfed throughout its life cycle as compared to *loh2* and other mutants. In addition, these mutant lines were also smaller when grown *in vitro* (figure 2). On the other side, *atr4* and *atr5* had slightly higher fresh weight when grown in vitro. To see if this was due to delayed growth and/or development, we analyzed the entry into different developmental stages of all atr mutants and compared the data with *loh2*. No statistically significant differences were observed during earlier stages from germination to rosette leaf stages (data not shown). However, *atr2* had late-

Table 1: The *atr2* mutant exhibits late flowering and delayed senescence.

	loh2	*atr1*	*atr2*	*atr3*	*atr4*	*atr5*	*atr6*	*atr7*	*atr8*	*atr9*
IE	29.2±2.1	30±2.3	36.1±1.9	30.7±2.5	28.3±1.4	30.1±2	32.4±1.7	31.5±2.9	30.4±1.3	32.5±1.9
FI	37.9±2.3	38.7±2.3	48.3±2.3	41.1±3.3	37.7±2.1	39.5±2.9	42.8±1.4	42±2.7	38.4±1.9	41.8±1.8
FC	62.6±3.2	61.1±2.7	78.5±4	71.1±4.7	66.2±3.7	62.9±2.7	72±4.2	64.4±5.8	65.6±3.9	67.9±3.9
S	77.9±2.2	79.8±1.3	89.5±2.9	81.1±1.9	80.9±1	80.7±1.6	85.1±2.8	80.1±2.1	79.9±2	80.6±1.6

loh2 and *atr* mutants were grown on soil under standard greenhouse conditions and the days of appearance of the late developmental stages recorded. Data are means ± SD of three biological repetitions. IE; inflorescence emergence, FI; flower initiation, FC; flowering completed, S; senescence.

emergence of inflorescence, late flowering and delayed senescence (table 1). These findings suggest that some of the genes involved in stress tolerance and PCD also play roles in plant growth and development/senescence.

3.2. atr mutants exhibit enhanced tolerance to oxidative stress

Earlier studies have indicated a clear link between AAL toxin and oxidative stress (Gechev et al., 2004). Furthermore, the first mutant isolated and characterized exhibited enhanced tolerance toward ROS-induced cell death (Gechev et al., 2008). To evaluate the oxidative stress tolerance of other *atr* mutants, *loh2* and the nine *atr* mutants, including *atr1* as a positive control, were germinated and grown on plant media supplemented with 5, 7 and 9 μM AT. The sensitivity of *loh2* to AT was comparable to that of its original wild-type background *A. thaliana* ecotype *Wassilewskija*. Under these conditions, all lines were clearly inhibited in growth, which was confirmed by fresh weight measurements (figure 3). However, while *loh2* developed necrotic lesions, bleached, lost its chlorophyll and eventually died within 2 weeks after germination (with first necrotic lesions and chlorophyll loss already visible 5 days after germination), some of the *atr* mutants did not exhibit such severe yellowing characteristic of *loh2* on media with AT. In particular, *atr1, atr2, atr7, atr8* and *atr9* stayed much greener and did not die even 1 month after AT treatment. This was confirmed by measurements of fresh weight and chlorophyll content (figure 3A and 3B). The mutants *atr1, atr2, atr7, atr8,* and *atr9* had much less pronounced reduction of fresh weight and chlorophyll loss compared to *loh2* on all three concentrations of AT. In addition to H_2O_2, oxidative stress and subsequent cell death can be imposed by other types of ROS as superoxide radicals or singlet oxygen (Op Den Camp et al., 2003; Vranova et al., 2002). For example, paraquat generates superoxide radicals by accepting electrons from PSI and transferring them to oxygen (Gechev et al., 2006). We tested *atr* mutants on media supplemented with 0.5, 1 or 1.5 μM paraquat and found that *atr1, atr2, atr7,* and *atr9* had enhanced tolerance to paraquat on all three concentrations. For example, the oxidative stress-tolerant mutants have much less fresh weight loss and retain more of their chlorophyll compared with *loh2* on media with 1.5 μM paraquat (figure 3C and 3D).

Table 2: Gene expression analysis of *loh2* and *atr* mutants exposed to AT-induced oxidative stress.

Gene Name	TAIR locus	*loh2*	*atr1*	*atr2*	*atr3*	*atr4*	*atr5*	*atr6*	*atr7*	*atr8*	*atr9*
High-affinity nitrate transporter	AT1G08090	7.29	2.95	6.4	50.2	27.4	11.7	44.9	45.2	1.11	5.8
Hsp17	AT3G46230	4.27	–2.17	130.8	6.4	8.41	11.2	57.4	–9.1	41.6	525
Extensin like	AT5G46890	–2.1	–16.7	–25	–2.4	–9.1	–10	–1.9	–2.1	–14.3	–2.6

loh2 and *atr* mutants were grown *in vitro* on MS media supplemented or not with 7 μM AT and samples for real-time PCR analysis were taken 4 days after germination before any visible stress symptoms. Positive values indicate higher expression in AT-treated plants (fold change, genes induced by oxidative stress), while negative values indicate lower expression in AT-treated plants (fold change, gene repressed by oxidative stress).

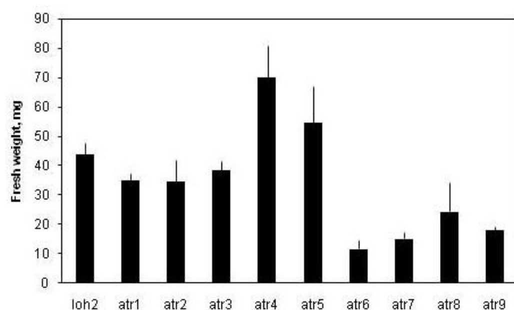


Figure 2: Fresh weight (FW) of *loh2* and *atr* mutants. Seedlings were grown for seven days on MS media under normal growth conditions (PPFD 100 μmol m^{-2} s^{-1}, 22°C) and fresh weight of the seedlings is calculated from three independent biological repetitions. Data are means ± SEM.

However, there was a gradation in the tolerance of the mutants to paraquat. Paraquat-induced growth inhibition and fresh weight loss was much more pronounced than AT-induced growth inhibition. According to the fresh weight data, *atr7* and *atr9* suffered less, followed by *atr1* and *atr2*. As for the chlorophyll content, *atr2*, *atr7* and *atr9* retained more pigments than the other mutants.

The nine *atr* mutants can be divided into two groups: mutants tolerant to AAL toxin only and mutants more tolerant to both AAL toxin and ROS generated by AT or paraquat. The first group may reflect genes with specific roles in AAL toxin stress responses, whereas the second group may contain genes situated in a more general cell death pathway, probably the converging path of AAL toxin, H_2O_2 and superoxide radical-induced PCD. Catalase deficiency has previously been shown to induce oxidative stress and subsequently cell death in a number of species, including tobacco and *Arabidopsis* (Dat et al., 2003; Vanderauwera et al., 2005). Using a reverse genetics approach, an oxoglutarate-dependent dioxygenase was implicated as a player in AT-induced cell death (Gechev et al., 2005). Because H_2O_2-induced cell death is believed to be a complex process involving many genes, the *atr* mutants can serve as a starting point for further exploring the complexity of oxidative stress-induced cell death.

3.3. Molecular analysis of mutants exposed to AT-induced oxidative stress

To further gain understanding of the oxidative stress tolerance, we performed

Table 3: Catalase activity in *loh2* and *atr* mutants under normal growth conditions and AT-induced oxidative stress.

	loh2	***atr1***	***atr2***	***atr3***	***atr4***	***atr5***	***atr6***	***atr7***	***atr8***	***atr9***
MS	0.2±0.02	0.3±0.05	0.2±0.06	0.2±0.03	0.2±0.03	0.2±0.04	0.2±0.04	0.2±0.07	0.3±0.04	0.2±0.03
MS + 7 µM AT	0.1±0.02	0.1±0.02	0.1±0.02	0.1±0.02	0.1±0.02	0.1±0.03	0.1±0.02	0.1±0.01	0.1±0.03	0.1±0.02

Seedlings were grown for 4 days under normal growth conditions (PPFD 100 µmol m^{-2} s^{-1}, 22°C) on MS media or on MS supplemented with 7 µM AT. Catalase activity is expressed in µmol H_2O_2 min^{-1} µg $protein^{-1}$. Data are means ± SD of three biological repetitions.

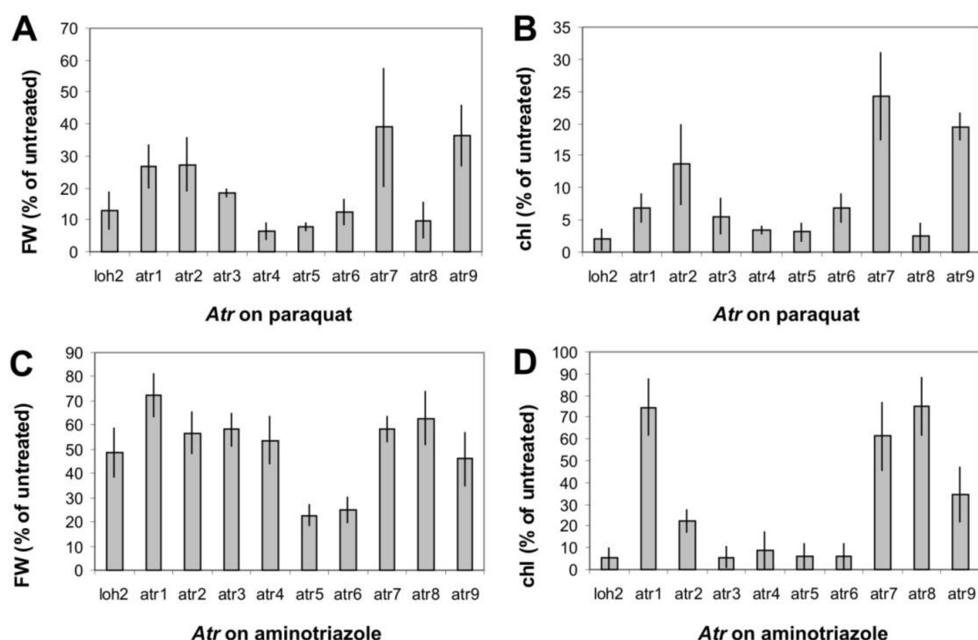


Figure 3: *atr* mutants and their tolerance to ROS-induced cell death. Seeds of nine *atr* mutants initially identified as more tolerant to AAL-toxin were plated on Murashige and Skoog (MS) media supplemented either with 1.5 µM paraquat (A, B) or with 7 µM aminotriazole (C, D) in order to assess their tolerance to cell death induced by superoxide radicals or hydrogen peroxide, respectively. Data represents the loss of fresh weight (FW) or chlorophyll (chl) of 10-day-old *loh2* and *atr* seedlings grown on media supplemented with paraquat or aminotriazole and compared with plants grown without paraquat and aminotriazole (percentage from untreated). Samples for the measurements were collected one week after germination. Data are means of three measurements ± SD.

real-time PCR analysis of gene expression with selected genes in the mutant lines exposed to AT-induced oxidative stress (table 2). A gene encoding *HIGH AFFINITY NITRATE TRANSPORTER* (At1g08090) was previously shown to be highly induced in both *loh2* and *atr1* in microarray experiments of AT treated plants (Gechev et al., 2008). Indeed, this gene was induced in all mutant lines (table 2), indicating that AT induced oxidative stress resulting in growth inhibition. The growth inhibition was confirmed by the repression of the *EXTENSIN* gene At5g46890 (table 2). Extensins

are known to play an essential role in cell expansion and their repression is consistent with growth inhibition. Earlier microarray analysis of AT-induced gene expression in *loh2* and *atr1* also revealed downregulation of the extensin gene (Gechev et al., 2008). It seems that oxidative stress results in a common response of all lines exhibited as a cessation of growth. However, the oxidative stress-tolerant lines, *atr1*, *atr2*, *atr7* and *atr9*, are able to somehow circumvent the growth inhibition and avoid AT-induced cell death. The heat shock encoding gene *HSP17* is not expressed under normal conditions at this stage of development, but is highly upregulated in *loh2* on AT-induced oxidative stress, as revealed by microarray analysis (Gechev et al., 2008). Interestingly, this upregulation was evident only for *loh2* and not *atr1*. Herewith, we confirm the induction of *HSP17* in *loh2* and the absence of induction in *atr1*. Furthermore, we show that this gene is induced in all other lines except *atr7* (table 2). The numbers even show downregulation of *HSP17* in *atr7*; however, taking into account the very low basal levels of expression, we can conclude that this gene is actually not expressed. As *atr7* is one of the mutants with high level of tolerance to AT, it seems that the mechanisms conferring oxidative stress tolerance may be different in *atr7* compared with other mutants. Because AT inhibits catalase and this leads to subsequent elevation of H_2O_2 levels, we determined the catalase activity in *loh2* and *atr* mutants. Catalase activity was inhibited by AT in all lines, demonstrating that the oxidative stress tolerance of the *atr* mutants was not due to inability of AT to inhibit the calatase (table 3). This means that an initial accumulation of H_2O_2 serving as a signal for oxidative stress and cell death is given. This conclusion is indirectly supported also by the expression of the nitrate transporter and the extensin genes. In the mutants most tolerant to oxidative stress, catalase activities were significantly lowered to an extent comparable to *loh2*. The inhibition of catalase activity by AT in both *loh2* and *atr* mutants suggests that the mutations conferring oxidative stress tolerance may be interfering with the perception or/and transduction of the redox signal rather than H_2O_2 accumulation. Alternatively, the mutation may inactivate a gene essential for regulation or execution of the cell death program that is situated below the H_2O_2 perception and transduction, downstream in the signaling cascade. Interference with

other signaling molecules such as reactive nitrogen species also cannot be excluded. ROS and nitric oxide ($NO^{\bullet}$) interact to modulate together many cellular processes, including PCD (Gechev et al., 2006). H_2O_2-stimulated $NO^{\bullet}$ accumulation has recently been shown to be executed by the prohibitin gene PHB3 (Wang et al., 2010). Mutation in PHB3 abort $NO^{\bullet}$ accumulation but does not affect H_2O_2 signaling. Further substantiating the link between ROS and $NO^{\bullet}$ during cell death is the recent positional cloning of *PARAQUAT RESISTANT2* gene in *Arabidopsis*, which encodes an S-nitrosoglutathione reductase (Chen et al., 2009). Positional cloning of the *atr* mutants will help to understand the intricate mechanisms of cell death tolerance in *Arabidopsis*.

4. Summary

Nine mutants with enhanced tolerance to AAL toxin have been isolated. Some of the mutants also exhibit enhanced tolerance to ROS-induced cell death triggered by AT or/and paraquat. Mutants with increased tolerance to all cell death stimuli may represent genes from a converging or downstream cell death pathway, whereas mutants that exhibit enhanced tolerance to the AAL toxin only may represent genes involved specifically in AAL toxin stress responses. Six of the mutants were smaller in size both when grown *in vitro* and on soil. Furthermore, some of them had altered leaf shape and one of them exhibited delayed senescence. Taken together, these results indicate that some of the genes responsible for oxidative stress tolerance and cell death may also be involved in modulating plant growth, development and senescence. Evaluation of oxidative stress tolerance together with expression analyses of gene markers for oxidative stress and cell expansion reveal that AT-induced oxidative stress leads to growth inhibition in all mutants, but AT-induced cell death is overcome in *atr1*, *atr2*, *atr7* and *atr9*. The different gene expression pattern of *atr7* suggests a different mechanism of stress tolerance.

Acknowledgements

The authors wish to thank to N. Mehterov for technical assistance. This work has been financially supported by NSF of Bulgaria, contracts DO02-281, DO02-330, and G-5, University of Plovdiv grant PC09-BF-063, and Higher Education Commission (HEC) of Pakistan.

References

1. Apel K, Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol **55:** 373-399.
2. Asai T, Stone JM, Heard JE, Kovtun Y, Yorgey P, Sheen J, Ausubel FM (2000) Fumonisin B1-induced cell death in *Arabidopsis* protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. Plant Cell **12:** 1823-1835.
3. Bach L, Michaelson LV, Haslam R, Bellec Y, Gissot L, Marion J, Da Costa M, Boutin JP, Miquel M, Tellier F, Domergue F, Markham JE, Beaudoin F, Napier JA, Faure JD (2008) The very-long-chain hydroxyl fatty acyl-CoA dehydratase PASTIC-CINO2 is essential and limiting for plant development. Proc Natl Acad Sci USA **105:** 14727-14731.
4. Brandwagt BF, Mesbah LA, Takken FLW, Laurent PL, Kneppers TJA, Hille J, Nijkamp HJJ (2000) A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp *lycopersici* toxins and fumonisin B-1. Proc Natl Acad Sci USA **97:** 4961-4966.
5. Chen M, Markham JE, Dietrich CR, Jaworski JG, Cahoon EB (2008) Sphingolipid long-chain base hydroxylation is important for growth and regulation of sphingolipid content and composition in *Arabidopsis*. Plant Cell **20:** 1862-1878.
6. Chen R, Sun S, Wang C, Li Y, Liang Y, An F, Li C, Dong H, Yang X, Zhang J, Zuo J (2009) The *Arabidopsis* PARAQUAT RESISTANT2 gene encodes an S-nitrosoglutathione reductase that is a key regulator of cell death. Cell Res **19:** 1377-1387.
7. Dat JF, Pellinen R, Beeckman T, van de Cotte B, Langebartels C, Kangasjarvi J, Inzè D, Van Breusegem F (2003) Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. Plant J **33:** 621-632.
8. Gadjev I, Stone JM, Gechev T (2008) Programmed cell death in plants: new insights into redox regulation and the role of hydrogen peroxide. Int Rev Cell Mol Biol **270:** 87-144.
9. Gechev T, Ferwerda M, Mehterov N, Laloi C, Qureshi MK, Hille J (2008) *Arabidopsis* AAL-toxin-resistant mutant *atr1* shows enhanced tolerance to

programmed cell death induced by reactive oxygen species. Biochem Biophys Res Commun **375:** 639-644.
10. Gechev T, Gadjev I, Van Breusegem F, Inzè D, Dukiandjiev S, Toneva V, Minkov I (2002) Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. Cell Mol Life Sci **59:** 708-714.
11. Gechev T, Willekens H, Van Montagu M, Inzè D, Van Camp W, Toneva V, Minkov I (2003) Different responses of tobacco antioxidant enzymes to light and chilling stress. J Plant Physiol **160:** 509-515.
12. Gechev TS, Gadjev IZ, Hille J (2004) An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity of programmed cell death in plants. Cell Mol Life Sci **61:** 1185-1197.
13. Gechev TS, Hille J (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol **168:** 17-20.
14. Gechev TS, Minkov IN, Hille J (2005) Hydrogen peroxide-induced cell death in *Arabidopsis*: Transcriptional and mutant analysis reveals a role of an oxoglutarate-dependent dioxygenase gene in the cell death process. IUBMB Life **57:** 181-188.
15. Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays **28:** 1091-1101.
16. Op Den Camp RG, Przybyla D, Ochsenbein C, Laloi C, Kim CH, Danon A, Wagner D, Hideg E, Gobel C, Feussner I, Nater M, Apel K (2003) Rapid induction of distinct stress responses after the release of singlet oxygen in *Arabidopsis*. Plant Cell **15:** 2320-2332.
17. Shi LH, Bielawski J, Mu JY, Dong HL, Teng C, Zhang J, Yang XH, Tomishige N, Hanada K, Hannun YA, Zuo JR (2007) Involvement of sphingoid bases in mediating reactive oxygen intermediate production and programmed cell death in *Arabidopsis*. Cell Res **17:** 1030-1040.
18. Spassieva SD, Markham JE, Hille J (2002) The plant disease resistance gene *Asc-1* prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death. Plant J **32:** 561-572.
19. Teufel A, Maass T, Galle PR, Malik N (2009) The longevity assurance homologue of yeast lag1 (Lass) gene family. Int J Mol Med **23:** 135-140.
20. Vanderauwera S, Zimmermann P, Rombauts S, Vandenabeele S, Langebartels C, Gruissem W, Inzè D, Van Breusegem F (2005) Genome-wide analysis of hydrogen peroxide-regulated gene expression in *Arabidopsis* reveals a high light-induced transcriptional cluster involved in anthocyanin biosynthesis. Plant Physiol **139:** 806-821.

21. Vranova E, Atichartpongkul S, Villarroel R, Van Montagu M, Inzè D, Van Camp W (2002) Comprehensive analysis of gene expression in *Nicotiana tabacum* leaves acclimated to oxidative stress. Proc Natl Acad Sci USA **99:** 10870-10875.
22. Wang Y, Ries A, Wu K, Yang A, Crawford NM (2010) The *Arabidopsis* prohibitin gene PHB3 functions in nitric oxide-mediated responses and in hydrogen peroxide-induced nitric oxide accumulation. Plant Cell **22:** 249-259.
23. Winer J, Jung CKJ, Shackel I, Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem **270:** 41-49.
24. Zheng H, Rowland O, Kunst L (2005) Disruptions of the *Arabidopsis* enoyl-CoA reductase gene reveal an essential role for very-long chain fatty acid synthesis in cell expansion during plant morphogenesis. Plant Cell **17:** 1467-1481.

Chapter 4

Isolation and characterization of genes from *atr2* and *atr7* that confer enhanced tolerance to oxidative stress in *Arabidopsis thaliana*

Muhammad Kamran Qureshi[1]

Neerakkal Sujeeth [1, 2]

Tsanko S. Gechev[2]

Jacques Hille[1]

[1] Department Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands

[2] Department of Plant Physiology and Plant Molecular Biology, University of Plovdiv, 24 Tsar Assen str., Plovdiv 4000, Bulgaria

Abstract

The AAL-toxins (*Alternaria alternata* f. sp. *lycopersici*-toxins) can trigger programmed cell death (PCD) in *Arabidopsis* when a gene homologous to the tomato *Asc* (*Alternaria* stem canker) gene is knocked out. This *Arabidopsis* mutant is designated as *loh2* (LAG one homologue2). AAL-toxins-induced PCD is triggered by the disruption of sphingolipid metabolism and accumulation of reactive oxygen species (ROS). Genetic screening of the progeny of chemically mutagenized *loh2* seeds resulted in the isolation of nine AAL-toxins resistant (*atr*) mutants. These mutants also exhibited tolerance to ROS (Gechev et al. in Biochem Biophys Res Commun 375: 639-644, 2008, Chapter 2; Qureshi et al. in Acta Physiol Plant 33: 375-382, 2011, Chapter 3). Map-based cloning and sequence data analysis of the affected genes in two mutants, *atr2* and *atr7*, indicated that the mutations are in two uncharacterized genes At5g19210 and At5g21280, respectively. The *atr2* mutation leads to an amino acid change from proline to serine and/or serine to asparagine at two different locations in the gene. The *atr7* mutation results in a stop codon in the coding region of the gene. A DNA and protein sequence analysis of these genes indicated that At5g19210 has homology to DEAD-box RNA helicase proteins while At5g21280 encodes a hydroxyproline-rich glycoprotein.

Key words: AAL-toxins, programmed cell death, reactive oxygen species, map-based cloning; DEAD-box RNA helicase protein; hydroxyproline-rich glycoprotein

1. Introduction

Pathogens can induce programmed cell death (PCD) at the site of infection. The process is known as the hypersensitive response (HR) and occurs in incompatible plant-pathogen interactions. The HR allows the plant to restrict the pathogen by producing an inhospitable environment around the pathogen and limiting its spread to other plant parts. Necrotrophic pathogens feed on dead plant tissues by secreting toxins and inducing PCD in healthy tissues at the site of infection (Van Breusegem and Dat, 2006). One such example, *Alternaria alternata*, has the ability to trigger PCD in tomato by secreting host-specific *Alternaria alternata* f. sp. *lycopersici*-toxins (AAL-toxins). AAL-toxins disrupt the sphingolipid biosynthesis pathway by inhibiting the activity of ceramide synthase. Sphingolipids are key components of the endomembrane system. The enzyme, ceramide synthase, is involved in the biosynthesis of complex sphingolipids from sphinganine. Inability of the cells to synthesize certain complex ceramides, due to inactivity of one of the ceramide synthases, results in an increase in free sphingoid bases and can lead to PCD. A single gene, *Alternaria stem canker* (*Asc*), is responsible for AAL-toxins tolerance in tomato (Brandwagt et al., 2000; Spassieva et al., 2002). A knockout of a gene (LAG one homologue2; *loh2*) homologous to the *Asc* gene, in AAL-toxins insensitive *Arabidopsis*, was identified. AAL-toxins treatment of this *Arabidopsis* knockout results in the induction of PCD in the mutant plants. Microarray analysis of AAL-toxins treated *loh2* identified a set of early induced genes associated with reactive oxygen species (ROS) and ethylene, suggesting a role of ROS and ethylene in the induction of PCD (Gechev et al., 2004).

Previously, genetic screening of the progeny of *loh2* treated with ethyl methanesulfonate (EMS) resulted in the isolation of nine mutants. These mutants exhibited enhanced tolerance to AAL-toxins compared to *loh2*. Mutants were designated as AAL-toxin resistant, *atr* mutants (*atr1*-*atr9*). The *loh2* mutant develops necrotic symptoms at 20 nM and death at 40 nM concentration of AAL-toxins, unlike wild type *Arabidopsis* plants which are capable of tolerating 200 nM AAL-toxins. The *atr* mutants are able to tolerate 40 nM of AAL-toxins. In addition,

all the nine *atr* mutants were evaluated for ROS-induced stress tolerance and plant developmental processes. Some of these mutants are tolerant to PQ and/or to AT as well. PQ can induce oxidative stress in chloroplasts, whereas AT is a specific inhibitor of catalases. The analysis of mutants at different developmental stages indicated that some mutants had an altered phenotype. Six of the mutants were reduced in size compared to *loh2*. In addition, *atr2* and *atr7* had a higher fresh weight under PQ and AT induced oxidative stress and remained viable up to one month after AT treatment. However, both mutants had reduced fresh weight, smaller leaves and a reduced plant size compared to *loh2* under normal growth conditions. Additionally, *atr2* exhibited a late inflorescence emergence, late flowering and delayed senescence (Qureshi et al., 2011).

Here, we report the molecular isolation of two genes of the *atr* mutants, *atr2* and *atr7*, that are supposed to cause the phenotype. The mutant genes responsible for the altered phenotype were identified through map-based cloning and subsequent sequencing of the mapped region. Map-based cloning is the process to identify the position of the gene by measuring the genetic linkage of molecular markers with their known position on the genome (as described by Chen et al., 2009; Shirzadian-Khorramabad et al., 2010; Yoshida et al., 2002). Genetic screening of the F_2 population of a cross between wild type and either *atr2* or *atr7* indicated that the affected genes in both *atr2* and *atr7* segregate as a monogenic trait. The map-based cloning and sequence analysis of the mapped region revealed that both mutations are in two different uncharacterized genes located on chromosome V.

2. Materials and methods

2.1. Plant material and growth conditions

The *Arabidopsis thaliana* ecotype Colombia (Col-0) together with *atr2* and *atr7* were used in this study. Plants were grown on soil under standard growth conditions; 14 h light/10 h dark period, photosynthetic photon flux density 100 µmol m^{-2} s^{-1}, 22°C and a relative humidity of 70%. An organic-rich γ-ray radiated soil from Hortimea Groep, Elst, The Netherlands was used as a nutrient source. Seeds of

the wild type and mutants plants were plated on petri dishes with Murashige and Skoog (MS) salts containing vitamins, 1% sucrose and 0.8% agar supplemented with 1.5 µM PQ. MS media without 1.5 µM PQ were used for the control experiments. Seeds used for the experiment were surface sterilized with gaseous chlorine derived from sodium hypochlorite and 4 ml concentrated hydrochloric acid in a closed glass vessel. Seed germination was carried out in the climate room with a 16 h light/8 h dark period with a temperature of 22°C and a relative humidity of 65%. The light intensity was fixed at 120 µmol m^{-2} s^{-1}.

2.2. DNA isolation and genetic mapping of *atr2* and *atr7* mutants

Two independent F_2 populations generated from a cross between Col-0 and *atr2*/*atr7* were germinated on MS media supplemented with 1.5 µM PQ for seven to twelve days. The PQ-tolerant progenies were selected for genetic mapping. The selected plants were transferred to MS media without PQ for a few days before transferring to pots containing soil. The DNA was isolated from the leaves of the tolerant plants at an appropriate stage using the SHORTY method (http://www.biotech.wisc.edu/NewServicesandResearch/Arabidopsis/FindingYourPlantIndex.html). The *atr2* and *atr7* mutations were mapped roughly onto chromosome V by using SSLP (Simple sequence length polymorphism) markers from the TAIR database (The *Arabidopsis* information resource; www.arabidopsis.org). Larger F_2 populations of about 952 and 604 PQ-tolerant mutant plants for *atr2* and *atr7* respectively were then analyzed for fine mapping. The mutations in *atr2* and *atr7*, were mapped using SSLP, InDel (Insertion/Deletion) and SNP (Single-nucleotide polymorphism) markers (supplementary table 1). Potential SNPs were selected by randomly sequencing a 1 kb region of *loh2* containing the two mutations. The SNP markers were designed using the Web SNAPER program (Drenkard et al., 2000).

2.3. Genomic DNA isolation, sequencing and data analysis

Isolation of nuclear DNA was carried out for *atr2*, *atr7* and *loh2*. To minimize chloroplast and mitochondrial DNA contamination the FLORACLEAN™

plant DNA isolation kit (MP Biomedicals, Santa Ana, CA) was used. Genomic DNA from each of the mutants was pooled together and paired end sequencing was carried out using the Illumina analysis pipeline (Illumina, San Diego, CA) according to the manufacturer's protocol. The sequence contigs of ~200 bp obtained for *atr2*, *atr7* and *loh2* were then separately aligned to the Col ecotype reference genome sequence available in the database (GenBank accessions: Chromosome 1, NC_003070, Chromosome 2, NC_003071, Chromosome 3, NC_003074, Chromosome 4 NC_003075, and chromosome 5, NC_003076). The SNP list was generated in *atr2*, *atr7* and *loh2* by using the Col genome sequence as reference with the help of CLC-Bio software.

3. Results

3.1. Map-based cloning of *atr2* and *atr7*

The original *loh2* mutant is in a Wassilewskija (Ws) background. With the aim to identify the *atr2* and *atr7* mutations using a map-based cloning approach, we crossed the mutant plants with the Col-0 ecotype. F_2 populations were generated and homozygous *atr2*/*atr7* plants were isolated by testing the progeny on MS media supplemented with 1.5 µM PQ. Both *atr2*/*atr7* are able to tolerate 1.5 µM PQ compared to *loh2*, which dies under these conditions (Qureshi et al., 2011). The screening data indicates that both mutations segregate in a 3 (susceptible):1 (tolerant) Mendelian fashion (table 1). Initially, 50 PQ tolerant plants were selected for *atr2* and *atr7* and these were used for rough mapping. Interestingly, *atr2* and *atr7* were mapped to a common region between SSLP markers, CA72 and NGA139, comprising a region of about 4000 kb on chromosome V. The results offered the possibility that *atr2* and *atr7* were potential alleles. A cross between *atr2* and *atr7* negated the previous theory as the progeny was found to be PQ susceptible (data not shown). A large PQ tolerant population was developed for *atr2* (952) and *atr7* (604) in order to map the genes to a region less than 1cM (centiMorgan) or ~ 100 kb. Fine-mapping of the two plant populations was conducted with a combination of SSLP, SNP and InDel markers. The analysis delimited the *atr2* and

atr7 locus within a region of approximately 100 kb (figure 1).

Table 1: Genetic segregation of the *atr2* and *atr7* phenotype. Plants were screened on 1.5 µM PQ.

	F_2 Segregation		
	Sensitive	**Resistant**	**Ratio**
Col X *atr2*	3031	952	3.2:1
Col X *atr7*	2241	668	3.3:1

3.2. Genome sequencing and identification of the *atr2* mutation

Analysis using the TAIR database predicted 30 genes in the 100 kb region containing the *atr2* mutation. T-DNA knockouts were obtained for the candidate genes and germinated on MS media supplemented with PQ (supplementary table 2). Seedlings were grown for seven days in order to observe any change of phenotype. However, none of the available T-DNA knockouts produced a phenotype similar to that of *atr2* (data not shown). Therefore, whole genome sequencing of *loh2* and *atr2* was conducted using the Illumina sequence analyzer in order to find the candidate gene for *atr2*. EMS induces a C/G to T/A transition in more than 99% of the cases (Greene et al., 2003).

Therefore, an EMS induced single base pair change was assumed for the candidate gene in the mapped region. A list of SNPs was produced for *loh2* and *atr2*. After comparing the *loh2* and *atr2* lists, a total of 90 SNPs were found in the coding sequence of different genes in the region of interest. Out of these, 17 SNPs with C/G to T/A transitions were identified (data not shown). The examination of the 17 SNPs identified 2 potential C/G to T/A mutations, 3 base pair apart in the At5g19210 coding region.

The DNA and amino acid sequence of At5g19210 was aligned with genes of other plant species in order to find homology among different plant species (table 2). The sequence homology ranged from 66% to 42% at the DNA and 64% to 21% at the protein level among the species. In addition, protein sequence analysis identified seven motifs similar to that of DEAD-box RNA helicase proteins in the gene of interest and other comparable genes (figure 3). However, two DEAD-box

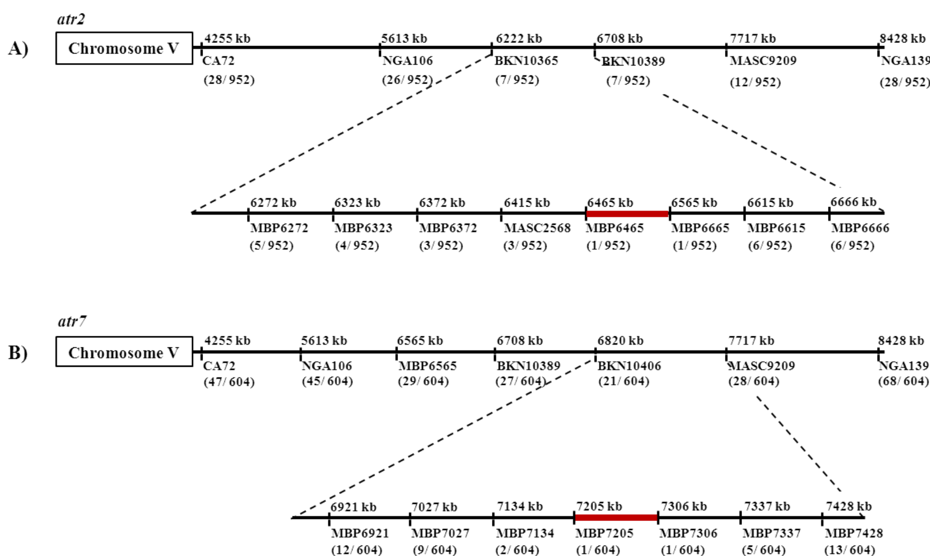


Figure 1: A schematic representation of a section of the *Arabidopsis thaliana* chromosome V covering a region in-between 4255 kb and 8428 kb. A) Representation of a region of the chromosome containing the *atr2* mutation marked by a red bar. The SSLP marker CA72, NGA106 and NGA139, InDel markers MBP6565 and 6615, and SNP markers BKN10365, 10389, MASC2568, 9209, MBP6272, 6323, 6372, 6465 and 6666 along with their position are indicated on the chromosome. The number of recombinants is also indicated. For example, (28/952) indicates 28 recombinants, from a total of 952, at approximate position of 4255 kb after analysis with marker CA72. B) Representation of a region of the chromosome containing the *atr7* mutation marked by a red bar. The SSLP marker CA72, NGA106 and NGA139, InDel markers MBP6565, 6921 and 7428, and SNP markers BKN10389, 10406, MASC9209, MBP7027, 7134, 7205, 7306 and 7337 along with their position are indicated on the chromosome. The number of recombinants is also indicated.

protein motifs were not found in all the genes analyzed. Apart from the presence of seven DEAD-box protein motifs, the *atr2* protein has sequence similarity with amino acid sequences of the compared genes in other parts of the protein sequence. Thus, it might be possible that this gene is conserved among different plant species.

3.3. Detection of the *atr7* mutation

Search results from the TAIR database predicted 44 genes in the region of 100 kb. The germination of T-DNA knockouts on MS media supplemented with PQ was

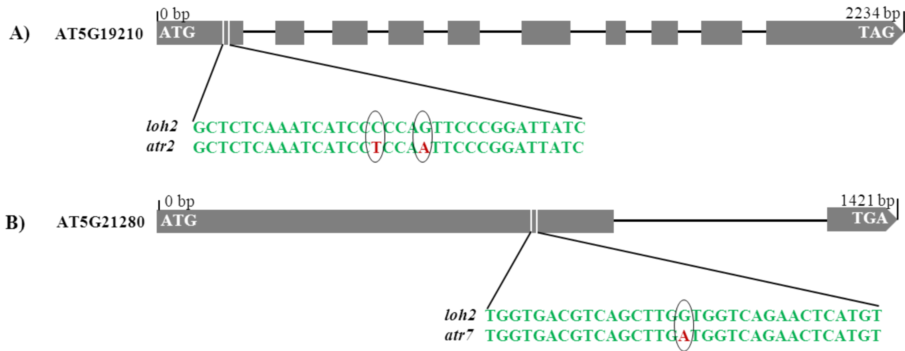


Figure 2: A genomic representation of the *atr2* and *atr7* candidate genes. The blocks indicate exons with introns in-between, represented by bars. A) the At5g19210 gene with its start site and stop codon. A fragment of the genomic sequence of *loh2* and *atr2* is shown with the sites of the two potential mutations for *atr2*, which results in amino acid changes, represented by encircling. B) the At5g21280 gene with its start site and stop codon. A fragment of the genomic sequence of *loh2* and *atr7* is shown with the site of the potential mutation for *atr7*, which results in a stop codon, represented by encircling.

unable to produce a phenotype, in seven days old seedlings, similar to that of *atr7* (supplementary table 2). Sequencing of the whole genome for *loh2* and *atr7* was conducted to find the mutation in the region of interest. Contigs obtained after sequencing *loh2* and *atr7*, were aligned to the Col reference sequence. A list of SNPs was generated from the aligned sequences. In total, 218 SNPs were found in the region of interest (data not shown). The list indicated 12 SNPs, with C/G to T/A shift in the coding region of different genes, as potential candidates for the *atr7* mutation. Out of these 12 SNPs, only one base pair change results in the premature termination of gene (At5g21280) expression.

The DNA and protein sequences of At5g21280 were compared with those of other plant species (table 3). Sequence homology ranging from 54% to 35% was observed in At5g21280 at the DNA and 44% to 35% at the protein level. Further analysis at the amino acid level identified the presence of proline rich motifs in At5g21280 and in other proteins analyzed (figure 4). In addition, the gene of interest shares sequence homology at the amino acid level with the protein sequence

of compared genes in other parts of the protein. The presence of proline rich motifs makes the gene similar to a hydroxyproline-rich glycoprotein (HRGP). A hydropathy profile was also developed for the amino acid sequence in order to identify hydrophilic and/or hydrophobic properties of the protein (figure 5). The result predicts that At5g21280 is not a membrane targeted protein.

4. Discussion

In the present study, two genes possibly responsible for the phenotype of *atr2* and *atr7* were identified using a map-based cloning approach. *atr2* and *atr7* were selected as they were more tolerant to oxidative stress compared to *loh2* and other *atr* mutants (*atr3*, *atr4* and *atr5*). Secondly, a cross between Col-0 and *atr2*/*atr7* resulted in 3:1 segregation of ROS tolerance (table 1). This indicates that both *atr2* and *atr7* are recessive alleles. Initial molecular analysis of *atr2* and *atr7* resulted in the identification of linkages within a 21 cM genomic region on chromosome V. Subsequent map-based cloning restricted the region around the two mutations to about 100 kb (figure 1). Further narrowing down the distance proved to be difficult due to a lack of recombinants.

4.1. *atr2* encodes At5g19210, a putative DEAD-box ATP-dependent RNA helicase

Analysis of the sequenced data indicated that the *atr2* phenotype most likely is caused by a mutation in At5g19210. This gene consists of 10 exons. The *atr2* mutant phenotype is potentially due to two single base pair changes, 3 base pairs apart, in the first exon (figure 2). The first base pair shift, C/G to T/A, results in an amino acid change from proline^{44} to serine^{44}. The second mutation results in a change of amino acid from serine^{45} to asparagine^{45}.

We also analyzed the T-DNA insertion mutants of At5g19210. The T-DNA insert in the exon region leads to the possible loss of gene function. However, the T-DNA knockout failed to produce a phenotype similar to *atr2*. Therefore, it can be argued that it is the amino acid change and not the knockout of the gene that caused

Table 2: The percentage similarity observed at the DNA and protein level in *atr2* (At5g19210) and other plant species.

	DNA accession number	Protein accession number	% homology at DNA level	% homology at protein level
At5g19210 (Reference)				
Uncharacterized				
Medicago truncatula	NC_016410	XP_003610394	66	61
Glycine max	NC_016094	XP_003528676	63	64
Nicotiana tabacum	AF261021	AAG34873	61	60
Characterized				
Oryza sativa AIP1	NM_001049832.1	Q0JM17.2	42	21
Oryza sativa AIP2	NM_001049833.1	Q5JK84.1	44	21

DNA and protein alignments were carried out separately using the At5g19210 DNA and protein sequences as reference molecules.

the phenotype. Similar results were found in the case of the *Arabidopsis old3-1* mutant. The *old3-1* mutation is due to the substitution of glycine^{162} to glutamic acid^{162} and results in early leaf senescence (Shirzadian-Khorramabad et al., 2010).

A TAIR database search revealed that At5g19210 encodes a putative DEAD-box ATP-dependent RNA helicase. RNA helicases are involved in many cellular processes. These enzymes function in the unwinding of double-stranded RNAs and in the rearrangement of RNA secondary structure. In the process, the energy is derived from the hydrolysis of adenosine triphosphate. DEAD-box RNA helicases are a family of RNA helicases found both in prokaryotes and eukaryotes. The name DEAD-box is derived from the presence of a protein motif, D-E-x-D/H as abbreviated in one letter codes for the amino acids (as reviewed by Rock and Linder, 2004). In *Arabidopsis*, more than 50 members of the DEAD-box RNA helicases family have been identified (Aubourg et al., 1999; Boudet et al., 2001). Some of these DEAD-box RNA helicases family members have been characterized with reference to stress response (Gong et al., 2005; Kant et al., 2007).

In total, there are nine conserved motifs in DEAD-box proteins, motif Q, I, Ia, Ib, II-VI. Motifs Q, I and II are required for the binding and hydrolysis of ATP (Rock and Linder, 2004). The analysis of protein sequence revealed that At5g19210 contains motifs similar to those of DEAD-box proteins such as I, Ia and II to VI. However, At5g19210 lacks motif Q and Ib, which makes the protein different from the DEAD-box protein family (figure 3). The DEAD-box RNA helicase like proteins have also been predicted in other plants species. Alignment of the DNA and protein sequence of At5g19210 with those of uncharacterized genes in other plants species indicated 66% to 60% homology (table 2). Interestingly, motifs Q and Ib were also absent in the species investigated, predicting the existence of a common function among the genes (figure 3). Thus, it can be predicted that the aligned genes might play a role, similar to that of *atr2*, in the PCD pathway in other plant species.

Two rice DEAD-box ATP-dependent RNA helicases, characterized as API5-INTERACTING PROTEIN1 (AIP1) and AIP2, are involved in the PCD process regulating tapetum degeneration. These proteins do not share a higher homology with *atr2* at the DNA and protein level, but share 7 DEAD-box RNA helicase motifs. Both AIP1 and AIP2 interact with APOPTOSIS INHIBITOR5 (API5), a nuclear protein, during pollen development. Down-regulation of AIP1 and AIP2 or knockout of API5 results in suppression of the cysteine protease gene (CP1) and causes male sterility (Li et al., 2011). As *atr2* contains all the motifs as in the AIP1 and AIP2 proteins, it can be speculated that *atr2* might play a similar role like those of AIP1 and AIP2 during the PCD process.

4.2. A mutation in At5g21280, encoding a hydroxyproline-rich glycoprotein most likely, results in the *atr7* phenotype

Sequence analysis of the 100 kb region harbouring the *atr7* mutation resulted in the identification of the candidate gene At5g21280. Genome analysis indicated that this gene consists of two exon regions. A single base pair change, from C/G to T/A, in the first exon results in a stop codon^{227}. A phenotype comparable to that of the *atr7* mutant was not observed in mutants with a T-DNA

insertion in At5g21280. The T-DNA insertion was in the promoter region of the gene. An analysis of 1084 insertion mutants in 755 *Arabidopsis* genes studied in 648 publications revealed that a T-DNA insertion in the protein-coding region of a gene is effective in 86% of the situations, while the insertion in the promoter region results in complete knockouts in 41% of the cases (as reviewed by Wang, 2008). Therefore, it can be argued that the T-DNA insertion in At5g21280 might not result in a complete knockout.

Table 3: The percentage similarity observed at the DNA and protein level in *atr7* (At5g21280) and other plant species.

	DNA accession number	Protein accession number	% homology at DNA level	% homology at protein level
At5g21280 (Reference)				
Uncharacterized				
Populus trichocarpa	XM_002308471	XP_002308507	54	44
Ricinus communis	NW_002994306	XP_002516170	46	36
Glycine max	NC_016093	XP_003526088	35	35

DNA and protein alignments were carried out separately using the At5g21280 DNA and protein sequences as reference molecules.

At5g21280 is an uncharacterized gene, as indicated by the TAIR database. Analysis of the sequence at the DNA and protein level reveals that At5g21280 shows resemblance to putative genes in other plants species (table 3). Additionally, the *atr7* protein shows sequence homology to hydroxyproline-rich glycoproteins (HRGPs) (figure 4). The HRGPs belong to a cell wall glycoproteins superfamily. These proteins constitute a signal peptide, which permits them to be exported to the cell wall, and include a proline rich repetitive region (as reviewed by Josè-Estanyol and Puigdomènech, 2000). The resemblance of the *atr7* encoded protein with HRGPs is due to the presence of proline rich motifs in protein sequences, but the

analyzed protein lacks a signal peptide. Moreover, the hydropathy plot for the amino acid sequence of At5g21280 predicts that the protein is lacking trans-membrane domains (figure 5). This makes the gene of interest different, compared to other structural HRGPs reported. Apart from the presence of proline rich motifs, the *atr7* encoded protein has sequence homology with the amino acid sequence of predicted genes, indicating that the gene might be conserved among different plants species (figure 4). Therefore, an *atr7*-similar function can also be predicted for the homologous genes in the other compared species.

AAL-toxins treatment disrupts not only the biosynthesis of sphingolipids, it also triggers the activation of ROS-related genes preceding PCD in *loh2* (Gechev et al., 2004). ROS are produced as a by-product during photosynthesis and respiration and are among the key inducers of PCD. They can trigger either a protective mechanism or PCD depending upon their dose. The mutants studied provide a link between sphingolipids and ROS signaling in plants (as reviewed by Gechev and Hille, 2005; Gechev et al., 2006). For example, *atr* mutants isolated as AAL-toxins resistant mutants exhibit tolerance to ROS-induced PCD and both *atr2* and *atr7* are no exception (Gechev et al., 2008; Qureshi et al., 2011). Therefore, it is likely that there exists an interaction between sphingolipids and ROS signaling in the regulation of PCD.

In summary, earlier analyses have elucidated the role of *atr2* and *atr7* in PCD induced by AAL-toxins, PQ and AT. In this study, we present the molecular isolation of the *atr2* and *atr7* mutations utilizing a map-based cloning approach and subsequent sequencing and analysis of the mapped region. The study revealed that the *atr2* and *atr7* phenotypes are most likely due to the mutations of At5g19210 and At5g21280, respectively. However, complementation tests are required in order to confirm these notions.

Figure 3: Homology found in the DEAD-box RNA helicase-like protein encoded by At5g19210 (*atr2*) with those of other plant species. Multiple sequence alignments of the protein molecules were carried out and a sequence logo was then created from the conserved sequences using Geneious v5.5 software (Drummond et al., 2011). Amino acids are represented by separate colours based upon their chemical properties. The height of the logo is proportional to the frequency of occurrence of a given amino acid. The red bars represent motifs I (AxxGxGKT), Ia (PTRELx), II (DExD/H), III (SAx), IV (xIFV), V (ARGID) and VI (HRxGRxGR).

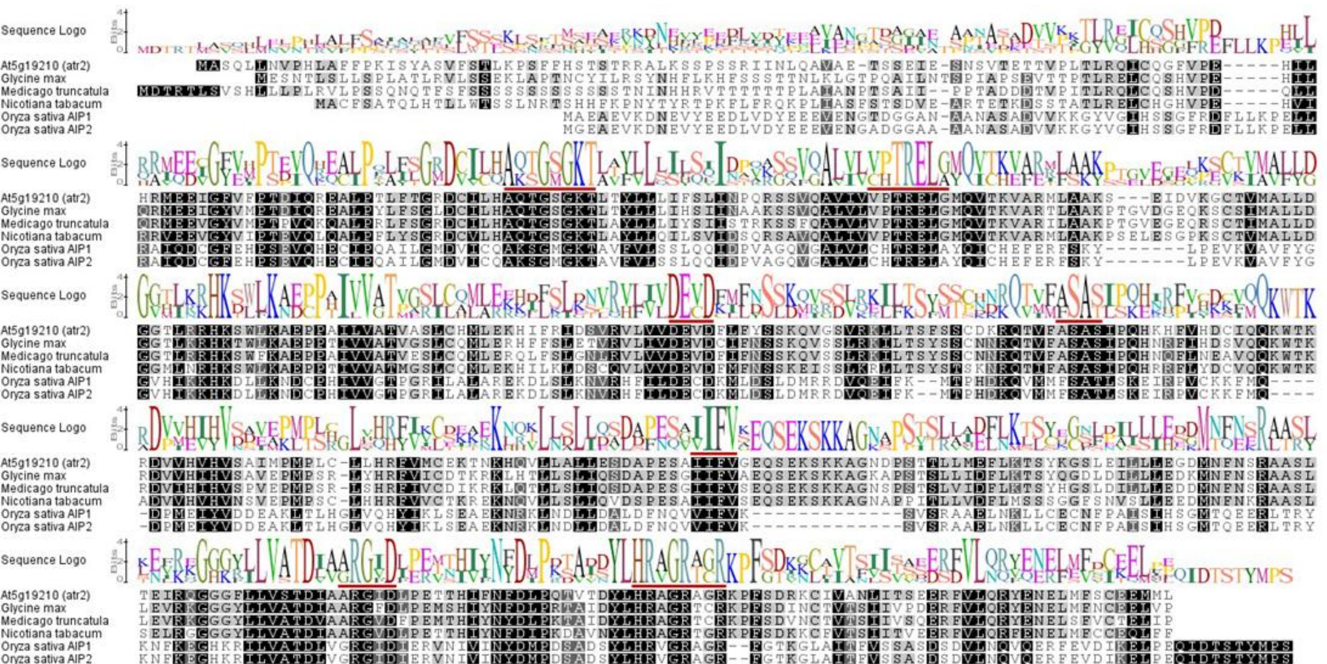
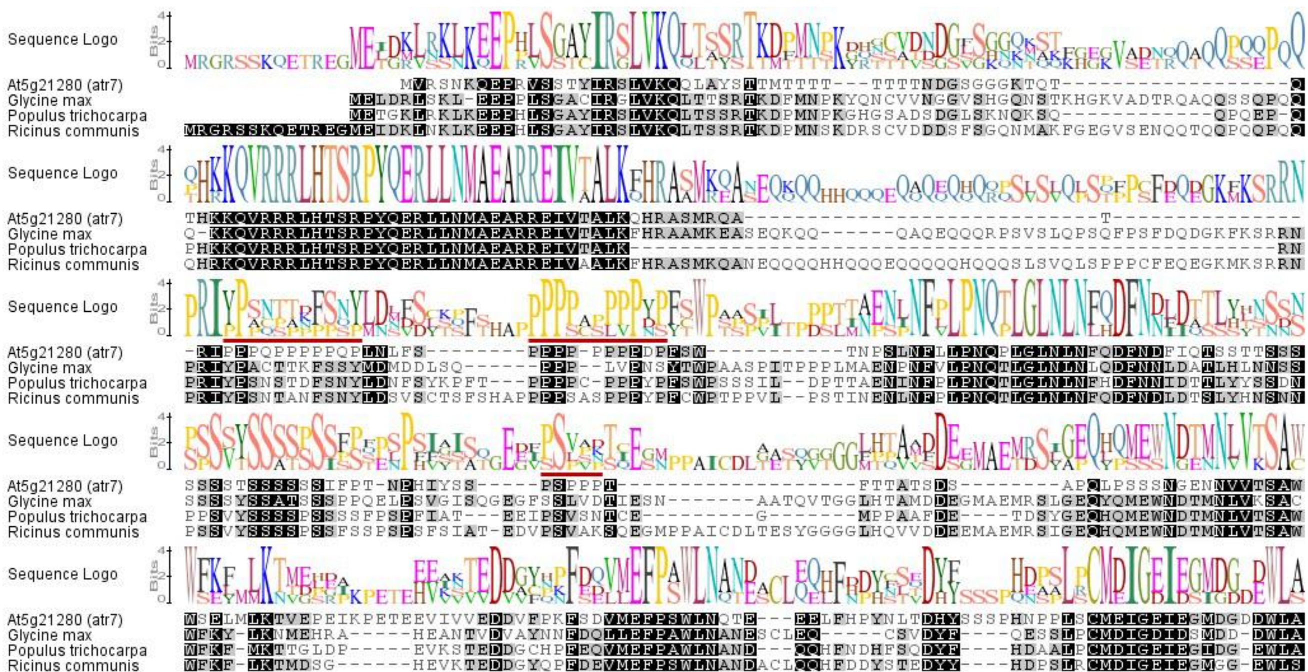


Figure 4: Homology found in the HRGP-like protein encoded by At5g21280 (*atr7*) with those of other plant species. Multiple sequence alignments of the protein molecules were carried out and a sequence logo was then created from the conserved sequences using Geneious v5.5 software (Drummond et al., 2011). Amino acids are represented by separate colours based upon their chemical properties. The height of the logo is proportional to the frequency of occurrence of a given amino acid. The red bars represent proline rich motifs in At5g21280 amino acid sequence.



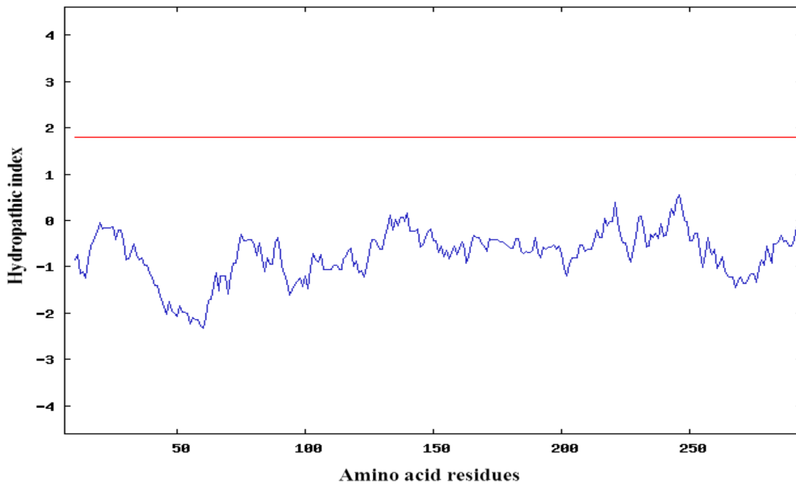


Figure 5: Hydropathy plot for the amino acid sequence of At5g21280 calculated according to the method of Kyte and Doolittle (1982) with an interval of 19 amino acid residues. The red line represents the trans-membrane indicator.

Supporting online material

http://www.rug.nl/staff/m.k.qureshi/supplementary_table_Chapter_4.pdf

Supplementary table 1: List of genes along with their knockouts in the predicted 100 kb region containing either the mutations in *atr2* or *atr7*.
Supplementary table 2: Different markers used for map-based cloning. These are SSLP (Simple sequence length polymorphism), InDel (Insertion/Deletion) and SNP (Single-nucleotide polymorphism) markers.

References

1. Aubourg S, Kreis M, Lecharny A (1999) The DEAD box RNA helicase family in *Arabidopsis thaliana*. Nucleic Acids Res **27:** 628-636.
2. Boudet N, Aubourg S, Toffano-Nioche C, Kreis M, Lecharny A (2001) Evolution of intron/exon structure of DEAD helicase family genes in *Arabidopsis*, *Caenorhabditis*, and *Drosophila*. Genome Res **11:** 2101-2114.
3. Brandwagt BF, Mesbah LA, Takken FLW, Laurent PL, Kneppers TJA, Hille J, Nijkamp HJJ (2000) A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp *lycopersici* toxins and fumonisin B1. Proc Natl Acad Sci USA **97:** 4961-4966.

4. Chen R, Sun S, Wang C, Li Y, Liang Y, An F, Li C, Dong H, Yang X, Zhang J, Zuo J (2009) The *Arabidopsis* PARAQUAT RESISTANT2 gene encodes an S-nitrosoglutathione reductase that is a key regulator of cell death. Cell Res **19:** 1377-1387.
5. Drenkard E, Richter BG, Rozen S, Stutius LM, Angell NA, Mindrinos M, Cho RJ, Oefner PJ, Davis RW, Ausubel FM (2000) A simple procedure for the analysis of single nucleotide polymorphisms facilitates map-based cloning in *Arabidopsis*. Plant Physiol **124:** 1483-1492.
6. Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2011) Geneious v5.5, Available from http://www.geneious.com.
7. Gechev TS, Gadjev IZ, Hille J (2004) An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity of programmed cell death in plants. Cell Mol Life Sci **61:** 1185-1197.
8. Gechev TS, Hille J (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol **168:** 17-20.
9. Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays **28:** 1091-1101.
10. Gechev TS, Ferwerda MA, Mehterov N, Laloi C, Qureshi MK, Hille J (2008) *Arabidopsis* AAL-toxin-resistant mutant *atr*1 shows enhanced tolerance to programmed cell death induced by reactive oxygen species. Biochem Biophys Res Commun **375:** 639-644.
11. Gong Z, Dong CH, Lee H, Zhu J, Xiong L, Gong D, Stevenson B, Zhu JK (2005) A DEAD box RNA helicase is essential for mRNA export and important for development and stress responses in *Arabidopsis*. Plant Cell **17:** 256-267.
12. Greene EA, Codomo CA, Taylor NE, Henikoff JG, Till BJ, Reynolds SH, Enns LC, Burtner C, Johnson JE, Odden AR, Comai L, Henikoff S (2003) Spectrum of chemically induced mutations from a large-scale reverse-genetic screen in *Arabidopsis*. Genetics **164:** 731-740.
13. Josè-Estanyol M, Puigdomènech P (2000) Plant cell wall glycoproteins and their genes. Plant Physiol Biochem **38:** 97-108.
14. Kant P, Kant S, Gordon M, Shaked R, Barak S (2007) STRESS RESPONSE SUPPRESSOR1 and STRESS RESPONSE SUPPRESSOR2, two DEAD-box RNA helicases that attenuate *Arabidopsis* responses to multiple abiotic stresses. Plant Physiol **145:** 814-830.
15. Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. J Mol Biol **157:** 105-132.

16. Li X, Gao X, Wei Y, Deng L, Ouyang Y, Chen G, Li X, Zhang Q, Wu C (2011) Rice APOPTOSIS INHIBITOR5 coupled with two DEAD-box adenosine 5'-triphosphate-dependent RNA helicases regulates tapetum degeneration. Plant Cell **23:** 1416-1434.
17. Qureshi MK, Radeva V, Genkov T, Minkov I, Hille J, Gechev TS (2011) Isolation and characterization of *Arabidopsis* mutants with enhanced tolerance to oxidative stress. Acta Physiol Plant **33:** 375-382.
18. Rocak S, Linder P (2004) DEAD-box proteins: the driving forces behind RNA metabolism. Nat Rev Mol Cell Biol **5:** 232-241.
19. Shirzadian-Khorramabad R, Jing HC, Everts GE, Schippers JH, Hille J, Dijkwel PP (2010) A mutation in the cytosolic O-acetylserine (thiol) lyase induces a genome-dependent early leaf death phenotype in *Arabidopsis*. BMC Plant Biol **10:** 80.
20. Van Breusegem F, Dat J (2006) Reactive oxygen species in plant cell death. Plant Physiol **141:** 384-390.
21. Wang YH (2008) How effective is T-DNA insertional mutagenesis in *Arabidopsis*? J Biochem Tech **1:** 11-20.
22. Yoshida S, Ito M, Nishida I, Watanabe A (2002) Identification of a novel gene HYS1/CPR5 that has a repressive role in the induction of leaf senescence and pathogen-defense responses in *Arabidopsis thaliana*. Plant J **29:** 427-437.

Submitted to Int J Mol Sci

Chapter 5

The zinc finger protein ZAT11 modulates paraquat-induced programmed cell death in *Arabidopsis thaliana*

Muhammad Kamran Qureshi[1]

Neerakkal Sujeeth [1, 2]

Tsanko S. Gechev[2]

Jacques Hille[1]

[1] Department Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands

[2] Department of Plant Physiology and Plant Molecular Biology, University of Plovdiv, 24 Tsar Assen str., Plovdiv 4000, Bulgaria

Abstract

Plants use programmed cell death (PCD) as a tool in their growth and development. PCD is also involved in defense against different kinds of stresses including pathogen attack. In both types of PCD reactive oxygen species play an important role. Microarray analysis of AAL-toxin and AT induced PCD in the *Arabidopsis* mutant *loh2* identified a number of genes highly regulated during various types of reactive oxygen species-induced PCD (Gechev et al. in Cell Mol Life Sci, 2004 61: 1185-1197; Qureshi et al. in Acta Physiol Plant 33: 375-382, 2011, Chapter 3). Eight of these genes were selected for further analysis and one of them, encoding the zinc finger protein ZAT11 (Zinc *Arabidopsis thaliana*11), exhibited an altered cell death phenotype. Two independent *zat11* mutants in a *loh2* genetic background showed enhanced tolerance to paraquat-induced oxidative stress and PCD but not to cell death triggered by AAL-toxin or AT. This indicates that ZAT11 is involved in an intricate oxidative stress-induced PCD network and the final outcome depends on ZAT11 interactions with other players specific for the particular types of oxidative stress.

Keywords: AAL-toxin; *Arabidopsis*; paraquat; programmed cell death; reactive oxygen species; ZAT11

1. Introduction

Programmed cell death (PCD) is a highly organized and genetically controlled suicidal process. PCD takes place during growth and development, e.g. embryo development, seed germination, tracheary element differentiation, anther development, self-incompatibility, formation of lace leaf shape and leaf senescence (as reviewed by Gunawardena, 2008; Ito and Fukuda, 2002). Additionally, PCD can occur in response to external stress such as high light/temperature, drought, salinity and during pathogen attack. Pathogen attack can induce PCD at the site of infection, a process known as the hypersensitive response (HR) in incompatible plant-pathogen interactions (as reviewed by Coll et al., 2011). Conversely, necrotrophic pathogens secrete toxins that trigger PCD in plant host cells. Thus, the dead cells serve as a source of nutrients for the pathogens (as reviewed by Van Breusegem and Dat, 2006).

Singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^•$) are collectively termed as reactive oxygen species (ROS). ROS act as important modulators of many biological functions. They are generated as by-products of two essential processes, photosynthesis and respiration. The production of ROS takes place mainly in the chloroplasts, mitochondria, in the cytosol and in peroxisomes (as reviewed by Halliwell, 2006). Excessive ROS produced under adverse environmental conditions can be either toxic or have signaling functions. The prolonged exposure to stress produces elevated concentrations of ROS resulting in damage to cellular proteins, pigments, lipids and DNA, which in turn leads to cell death. At lower concentrations, ROS orchestrate global changes in gene expression and, depending on the interaction with other signaling molecules, can result in stress acclimation or PCD (as reviewed by Gechev et al., 2006). A number of genes including *EXECUTER1* (reviewed by Gechev et al., 2006), are involved in the signaling events and the genetic control of ROS-modulated PCD.

Necrotrophic pathogens such as *Alternaria alternata* trigger PCD in plants by secreting host-selective *Alternaria alternata* f. sp. *lycopersici*-toxin (AAL-toxin). AAL-toxin hinders the activity of ceramide synthase (Sphinganine N-

acyltransferase), resulting in the inhibition of sphingolipid biosynthesis. Sphingolipids are essential components of the endomembrane systems in eukaryotes. Inhibition of ceramide synthase leads to the accumulation of ceramide precursors and depletion of sphingolipids. The outcome of this disturbed balance is cell death. The fungus utilizes dead cells as a nutrient source. Tolerance to AAL-toxin is governed by the *Alternaria stem canker* gene (*Asc*) in tomato. The *Asc* gene is homologous to the yeast Longevity Assurance Gene1 (LAG1) (Brandwagt et al., 2000; Spassieva et al., 2002). *Arabidopsis thaliana* is relatively insensitive to the effects of AAL-toxin treatment. A knockout in *Arabidopsis*, homologous to the *Asc* gene, was identified. This knockout, *loh2* (LAG one homologue2), rendered mutant plants sensitive to AAL-toxin. The AAL-toxin treated mutant plants show all the hallmarks of PCD. A microarray analysis of *loh2* treated with AAL-toxin identified a number of genes associated with the ROS and the ethylene signaling pathways. Additionally, genes encoding a variety of proteases were amongst the earliest induced genes, indicating a role of these proteases in PCD (Gechev et al., 2004).

The *loh2* mutant also demonstrates sensitivity to ROS-inducing herbicides such as 3-aminotriazole (AT) and methyl-viologen/paraquat (PQ) (Gechev et al., 2005; Gechev et al., 2008; Qureshi et al., 2011). AT is a specific inhibitor of antioxidant enzymes known as catalases. Catalases are involved in the detoxification of H_2O_2 inside the plant cell. Thus, the inhibition of catalase results in the accumulation of H_2O_2. Consequently, higher concentrations of H_2O_2 trigger PCD (Gechev et al., 2002; Zimmermann et al., 2006). PQ is herbicide, which has the ability to induce oxidative stress in chloroplasts. Application of PQ triggers elevation of ROS (O_2^-) in chloroplasts during photosynthesis by donating an electron to oxygen. The accumulation of ROS can subsequently lead to PCD. Tolerance to PQ can be achieved by blocking the ROS signaling pathway or by increased activity of antioxidant enzymes (Chen et al., 2009; Fujibe et al., 2004).

A massive array of genes were shown to be regulated during microarray studies of cell death caused by AAL-toxin or AT in *loh2* mutants. AAL-toxin triggered more than 3.5 fold modulation of 81 genes in *loh2*, seven hours after treatment (Gechev et al., 2004). Similarly, treatment of *loh2* with AT provoked expression changes of 219

genes with at least 5 fold increase or decrease within four days (Gechev et al., 2008). Some of the highly regulated genes were not previously linked to PCD and many of them are involved in plant development and associated with oxidative stress. Additionally, the function of some of these genes was not elucidated before (Gechev et al., 2008). The question remains whether these genes have an important role in the regulation of PCD. The aim of the present study is to establish whether there is a functional role of some of the regulated genes in relation to PCD. Eight genes were selected which are modulated at early time points during the cell death process triggered by AAL-toxin and/or AT treatment. The behaviour of mutants of those selected genes was studied in the *loh2* background.

2. Materials and methods

2.1. Selection of T-DNA insertion lines and establishing double mutants

Eight genes were selected from the microarray analysis on AAL-toxin and AT induced transcriptional alterations in *loh2* (table 1). Two independent T-DNA knockouts, having the T-DNA insertion at a different location in the gene, were selected from the TAIR database (www.arabidopsis.org) for each gene, in order to avoid potential artifacts. Seeds for the knockouts used in this study were obtained from the Nottingham *Arabidopsis* Stock Centre (NASC), University of Nottingham, UK (table 2). Homozygous plants from each T-DNA line were identified after screening the population, obtained from NASC, by utilizing PCR. The DNA was extracted from the leaves using the SHORTY method (http://www.biotech.wisc.edu/NewServicesandResearch/Arabidopsis/FindingYourPlantIndex.html).The PCR screening was conducted by using either of the gene-specific forward or reverse primer and a primer for the left border of the T-DNA insert. For WRKY 75, an RNAi line was exploited as a second mutant as the PCR product for the T-DNA insert proved difficult to be synthesized (Devaiah et al., 2007). The homozygous T-DNA mutants were crossed to *loh2*. The crosses were made using *loh2* as an ovule source and T-DNA mutant as a pollen source. The double mutants were screened by using the PCR method described earlier for the

identification of the T-DNA insert and *loh2*. For *loh2*, a 200 nM AAL-toxin infiltration method was also utilized as a second screening criterion. The AAL-toxin induces necrosis 3 days after treatment of homozygous *loh2* plants. The *loh2* phenotype is recessive, therefore the F_1 population of the cross between either of the homozygous T-DNA insert and *loh2* were unable to develop necrotic symptoms. The F_2 population was developed in order to obtain the *loh2* phenotype.

Total RNA was isolated from leaves of one month old plants using NucleoSpin RNA Plant kit (Macherey-Nagel, Germany). 1 µg of DNA-free total RNA was used for reverse transcription. Oligo (dT) primers were used for the reverse transcription. The relative expression of ZAT11 in single and double mutants, in relation to the endogenous control gene AAA ATPase, was assessed using qRT-PCR. The qRT-PCR was performed using a 7500 Real Time PCR system (Applied Biosystems). The following genes and corresponding primer pairs were used in the qRT-PCR analysis: ZAT11 (At2g37430), primer pairs ACCAACATACCGAGAGCCATAC and TTGACCTATGCCGTCTCATGTG; AAA ATPase (At3g28580), primer pairs GGCAATCTTTCTCGTTTTACCC and GCTCTATCGTCTTCCCTTCTCTC. The primers were designed from the coding regions of the genes. The PCR reaction mixture contained cDNA, 2x Power SYBR Green Master mix (Applied Biosystems, Foster City, CA) and 2 µM of each of the primer pairs for ZAT11 and AAA ATPase. Thermal cycling conditions were set at 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min followed by a dissociation stage in order to check for the presence of nonspecific PCR products. PCR products were run on a 1% agarose gel to assess the specificity of the amplicons. Gene expression (fold change) for *zat11* single and double mutants was compared with the wild type Col and calculated using the comparative threshold ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001). ZAT11 gene expression was normalized to that of the control AAA ATPase gene during the quantitative measurement.

2.2. Growth conditions and stress assays

Arabidopsis thaliana ecotypes Colombia (Col) and Wassilewskija (Ws) along

Table 1: Genes with a potential role in programmed cell death.

Sr. no.	AGI code	Description	7h, 24h, 48h, 72h AAL	AT	Remarks
1.	At2g37430	Putative C_2H_2-type zinc finger protein (ZAT11)	4.5, 3.0, 3.99, 6.72	2.56	Role in ROS-mediated PCD
2.	At5g13080	WRKY (AtWRKY 75) family transcription factor	3.8, 7.16, 68.0, 100.0	1.00	Induced during pathogen attack & oxidative stress
3.	At1g62300	AtWRKY 6	1.13, 1.58, 8.50, 8.07	1.27	Induced during pathogen attack & oxidative stress conditions
4.	At5g51060	Respiratory burst oxidase protein	3.5, 2.39, 18.4, 45.97	2.77	Involved in antioxidant metabolism & oxidative burst
5.	At1g32960	Subtilisin-like serine protease	2.1, 2.9, 12.9, 84.9	–1.15	Processing of bioactive cell wall peptides
6.	At4g28850	Xyloglucan endotransglycosylase	1.92, 1.0, 0.98, 1.0	–32.34	Loosening of cell wall for cell growth, repression may inhibit plant growth
7.	At2g18800	Xyloglucan endotransglycosylase	0.64, 1.1, 0.86, 0.75	–30.59	Loosening of cell wall for cell growth, repression may inhibit plant growth
8.	At4g26320	Arabinogalactan AGP 13	0.84, 0.6, 1.7, 0.75	–10.43	Regulator of cell growth & mediator of cell-cell intraction

Eight genes were selected from the studies conducted by Gechev et al. (2004) and Gechev et al. (2008) and their expression during the AAL-toxin- and AT-induced cell death given as fold change.

with *loh2* single and double mutant plants were germinated and grown on soil under the following growth conditions; 14 h light/10 h dark period, photosynthetic photon flux density 100 μmol m^{-2} s^{-1}, 22°C and relative humidity of 70%. An organic-rich γ-ray radiated soil was used from Hortimea Groep, Elst, The Netherlands. Four week old plants were used for the experiments. The plants were sprayed either with water, 15 μM PQ or 20 mM AT, whereas 200 nM AAL-toxin solution was infiltrated in the leaves using a syringe without a needle (Willekens et al., 1997). AAL-toxin solution was injected into the intercellular spaces through leaf mesophyll by applying gentle pressure to the syringe until the whole leaf is soaked with the solution. The soaked areas appear as dark green and this disappears within 1 hour after infiltration. Water infiltrated plants were used as controls. Samples were taken 7, 24, 48 and 72 hours after PQ/AT/AAL-toxin treatments. Cell death was determined as electrolyte leakage from the leaves by measuring the increase in conductivity. The leaf samples were immersed in ultrapure water for 30 minutes and the conductivity of the solution measured with an LF-91 conductivity meter. The samples were then autoclaved for a few minutes in the same solution and the conductivity was measured again as total conductivity. The increase in electrolyte leakage is expressed as percent of the total conductivity.

2.3. Cell death and chlorophyll measurement

Seeds of the wild type, single and double mutants were plated on petri dishes with Murashige and Skoog (MS) salts including vitamins, 1% sucrose and 0.8% agar containing either 0, 0.5, 0.1 μM PQ; 0, 5, 7, 9 μM AT; 0, 20, 40, 60 nM AAL-toxin solution. Surface sterilized seeds were used for experiments. The seeds were surface sterilized with gaseous chlorine derived from sodium hypochlorite with 4 ml of fuming hydrochloric acid in a closed glass container. The seeds were grown in a climate room with 16 h light/8 h dark period with a temperature of 22°C and relative humidity of 65%. The light intensity was set at 120 μmol m^{-2} s^{-1}. Stress tolerance was evaluated by measuring fresh weight and chlorophyll content. The samples were taken on the 7th day after germination in case of PQ treatment and on the 12th day after germination in case of AT and AAL-toxin treatment. Chlorophyll content was

Table 2: Mutants of the eight genes used in the functional studies to evaluate their potential role in programmed cell death.

Sr. no.	AGI code	Genomic Size (bp)	Knockout	Given Name	Type of insert	T-DNA insertion site
1.	At2g37430	868	SALK 110012	*zat11-1*	T-DNA	Promoter
			SALK 013996	*zat11-2*	T-DNA	Promoter
2.	At5g13080	1411	SALK 101367	*WRKY75-1*	T-DNA	Promoter
			RNAi line	*WRKY75-2*	RNAi insert	
3.	At1g62300	2597	SALK 059863	*WRKY6-1*	T-DNA	Promoter
			SALK 012997	*WRKY6-2*	T-DNA	Exon
4.	At5g51060	5148	SALK 018814	*RHD2-1*	T-DNA	Exon
			SALK 049688	*RHD2-2*	T-DNA	Promoter
5.	At1g32960	3344	SALK 107460	*SBT3.3-1*	T-DNA	Exon
			SALK 117861	*SBT3.3-2*	T-DNA	Exon
6.	At4g28850	1663	SALK 087890	*XTH26-1*	T-DNA	Exon
			SALK 124397	*XTH26-2*	T-DNA	Exon
7.	At2g18800	1354	SALK 057963	*XTH21-1*	T-DNA	Promoter
			SALK 124779	*XTH21-2*	T-DNA	Promoter
8.	At4g26320	371	WiscDsLox495C10	*AGP13-1*	T-DNA	Promoter
			SAIL 309 F04	*AGP13-2*	T-DNA	Exon

Two independent knockouts/RNAi lines were chosen for each gene.

quantified spectrophotometrically. The pigments from seedlings were extracted with 80% acetone at 4°C overnight. The absorption of chlorophyll a (*chyl a*) and b (*chyl b*) were measured at 663 and 647 nm. Chlorophyll content was calculated as microgram per milligram fresh weight (Inskeep and Bloom, 1985).

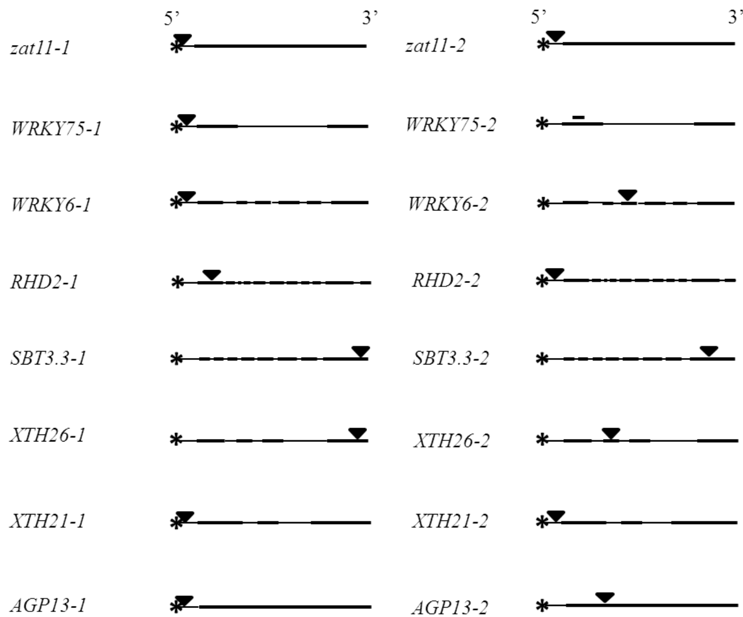


Figure 1: T-DNA insertion sites of the eight genes selected for the functional studies. The length of genes and their orientation have been normalized. The *— indicates the 5' to 3' promoter region of a gene. Two separate mutants for each gene, with independent T-DNA insertions, were utlized for the study. Relative position of each T-DNA insertion is depicted by an inverted triangle. In case of WRKY75-2, an RNAi insertion line was incorporated in the study.

3. Results

Based on the microarray analysis of AAL-toxin-induced cell death in *loh2*, 81 genes were noted as significantly regulated early in the process, 7 hours after AAL-toxin treatment. These genes play a crucial role not only during plant growth, development and defense but also in relation to ROS induced PCD (Gechev et al., 2004). Eight target genes, which are the entities of respective gene families, were

selected for further experiments. The selection is based upon a potential role of the selected genes as a regulatory element during PCD. Interestingly, the expression of the genes under investigation was also altered during AT triggered cell death in *loh2* (table 1) (Gechev et al., 2008). The list includes genes encoding a zinc finger protein (ZAT11), WRKY TFs (WRKY6 and WRKY75), xyloglucan endotransglycosylase/hydrolase (XTH21 and XTH26), respiratory burst oxidase protein (RHD2), subtilisin-like serine protease (SBT3.3) and arabinogalactan (AGP13). Some of the selected genes (WRKY75, RHD2 and SBT3.3) were highly induced, 72 hours after treatment with AAL-toxin. Two independent T-DNA mutants for each gene were used for the experiments. These mutants were either in the promoter or in the exon regions. Morphological differences were not observed between either of the single mutant and wild-type plants grown in soil under normal growth conditions (data not shown). Each of the independent mutants was crossed with *loh2*. The double mutants were screened as described earlier for further analysis. We conducted physiological experiments by utilizing different concentrations of either PQ, AT and AAL-toxin. These agents were added to MS media containing the seeds of wild types and mutants or sprayed on (PQ/AT), or infiltrated in (AAL-toxin) the leaves of four week old plants. Measurement of cell death was calculated as electrolyte leakage (supplementary table 3). Differences of significant values were not observed among double mutants and *loh2*.

3.1. Identification of *zat11* as modulator of paraquat-induced cell death

To further investigate the role of these eight genes, we subjected their respective knockout mutants as well as appropriate controls to three types of oxidative stress-induced cell death: cell death triggered by AAL-toxin, by the catalase inhibitor AT, and by paraquat (Gadjev et al., 2006; Gechev et al., 2004; Gechev et al., 2005).

3.1.1. *Alternaria alternata* f. sp. *lycopersici* (AAL)-toxin induced cell death

The cell death caused by AAL-toxin was light dependent and displayed the classic PCD symptoms in *loh2* (Gechev et al., 2004). Seeds from the knockouts and double mutants were germinated on MS media supplemented with 20, 40 or 60 nM AAL-toxin. Cell death was measured as loss of fresh weight and chlorophyll content. Decrease in fresh weight and chlorophyll content was observed with the increase in AAL-toxin concentrations (supplementary tables 1 & 2). Reduction in the seedling size was observable in case of *loh2* and all of the double mutants, 12 days after germination on MS media supplemented with 20 nM AAL-toxin. Partial to complete seedling bleaching, in the case of *loh2* and double mutants, was observed when applying 40 and 60 nM AAL-toxin treatments, respectively. The double mutants presented no significant differences when compared to *loh2* (supplementary tables 1 & 2). Single mutants as well as wild type seedlings developed no cell death symptoms when germinated on MS media supplemented with either of the 20, 40 and 60 nM AAL-toxin concentrations.

The *loh2* mutant plants exhibited necrotic lesions and increased electrolyte leakage, 72 hours after infiltration of 4 week old leaves with 200 nM AAL-toxin. In contrast, the leaves of the wild type and single mutants displayed no signs of necrosis (data not shown). The double mutants demonstrated similar death symptoms, comparable to *loh2*, 72 hours after AAL-toxin treatment (data not shown).

3.1.2. 3-Aminotriazole (AT) induced cell death

Seeds from the knockouts and double mutants were germinated on MS media supplemented with 5, 7 or 9 μM AT. *Arabidopsis thaliana* ecotype Col and Ws were used as wild type controls. The cell death was evaluated twelve days after germination. All the lines revealed reduction of fresh weight and chlorophyll contents with the increase of AT concentrations from 5 to 9 μM. Four rosette leaves were clearly visible, 12 days after germination on MS media supplemented with 5 μM AT. Yellowing of the leaves was observed starting from the petiole.

Additionally, root development was retarded compared to controls. A considerable increase in chlorophyll bleaching was observed in all the plants germinated in media containing 7 µM AT. Additional intensity of chlorophyll bleaching and root retardation was observed in the seedlings germinated on MS media supplemented with 9 µM AT. There were no significant differences among the mutants (supplementary tables 1 & 2).

Treatment of one-month-old plants by spraying 25 µM AT resulted in bleaching of the leaves. The direction of chlorophyll bleaching was from the leaf base towards the leaf margins (data not shown). The losses of viable cells were evaluated as an increase in the electrolyte leakage (supplementary table 3). This phenomenon was observed both among the mutants and the wild type. In addition, intensive bleaching was observed especially near the leaf base, 72 hours after treatment. However, there were no significant differences in single and double mutants.

3.1.3. Methyl-viologen/paraquat (PQ) induced cell death

The overall death was evaluated as the loss of fresh weight and chlorophyll contents seven days after germination of seedlings from single and double mutants. *Arabidopsis thaliana* ecotype Col and Ws were used as wild types. Significant reduction in fresh weight and chlorophyll contents was observed in the mutants as well as in the wild type grown in the media supplemented with 0.5 µM PQ. The seedling size was greatly reduced in all cases. Additionally, seedlings were unable to develop true leaves compared to controls, in which 2 rosette leaves were clearly observable after 7 days of germination. Death symptoms were observed at 1 µM PQ treatment. The seedlings were unable to develop beyond cotyledon leaves compared to controls (data not shown). No significant differences were observed in all double mutants compared to single T-DNA mutants and wild type plants (supplementary tables 1 & 2).

In case of a spraying experiment using 15 µM PQ on four weeks old plants, visible necrosis and bleaching symptoms were detected in all cases at different intensities as early as 24 hours after treatment. The necrosis symptoms were

observable as patches after 72 hours of PQ treatment (data not shown). The loss of viable cells was evaluated as increase in the electrolyte leakage (supplementary table 3). A significant amount of electrolyte leakage was characteristic for *loh2*.

Table 3. Relative expression analysis of *zat11* single and double mutants.

	Col	*zat11-1*	*loh2/ zat11-1*	*zat11-2*	*loh2/zat11-2*
Relative expression	1.00	7,8784E+75 ± 1,08882E+76	1,16396E+77 ± 1,60863E+77	4,1768E+68 ± 5,77247E+68	5,20733E+70 ± 7,19668E+70

Plant were grown on soil under standard climate room conditions. Leaf samples for qRT-PCR analysis were taken one month after germination. The values indicate relative expression compared to Col. Data represents $2^{-\Delta\Delta Ct}$ ± SD.

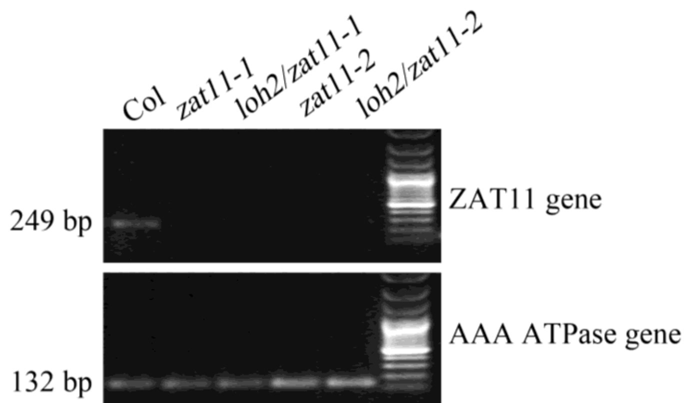


Figure 2: qRT-PCR profile for ZAT11 gene expression in wild type *Arabidopsis thaliana* ecotype *Columbia* and *zat11* knockout mutants. The ZAT11 expression is compared to the endogenous gene AAA ATPase.

Most of the single (T-DNA gene knockout) and double mutants (*loh2* X T-DNA gene knockout) did not show any significant change in the electrolyte leakage, similar to that recorded in either *loh2* or wild type plants (Col and Ws). Interestingly, a cross between *loh2* and each of the ZAT11 T-DNA insertion lines showed significant differences compared to their single mutants, when sprayed with 15 µM PQ (supplementary table 3, figure 3). Two independent T-DNA knockouts

zat11-1 and *zat11-2* in the promoter region of the ZAT11 gene were selected for the study. The mutants were further analysed for expression of the ZAT11 gene in *zat11-1* and *zat11-2* single and their respective double mutants with *loh2* at the RNA level. The quantitative real-time PCR (qRT-PCR) indicated that the ZAT11 gene is highly repressed in *zat11* single and double mutants (table 3; figure 2). Both the single mutants exhibited increased susceptibility towards PQ when compared to Col. However, electrolyte leakage was less than that in Ws and *loh2* (Figure 3). A cross between each of the knockouts (*zat11-1* and *zat11-2*) to *loh2* is represented as *loh2/zat11-1* and *loh2/zat11-2*. In both cases, a decrease in electrolyte leakage was observed in comparison with *loh2*, when treated with 15 µM PQ concentration. Interestingly, a similar reduction in electrolyte leakage was detected in both the double mutants compared to their respective *zat11* single mutants (figure 3).

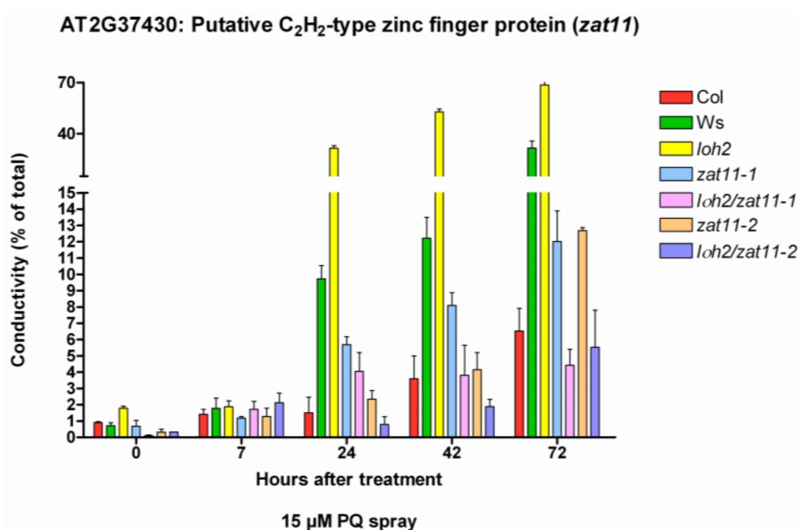


Figure 3: *zat11* knockouts demonstrate alterations in paraquat-induced cell death. Electrolyte leakage expressed as conductivity was used to evaluate the amount of cell damage and death. Four week old plants were sprayed with 15 µM PQ. Water treated plants were used as controls. Cell death was determined as the increase in conductivity and calculated as the percentage of total conductivity obtained after boiling the samples, at 7, 24, 48 and 72 hours after treatment. The data are represented as means of three biological replicates ± SD.

4. Discussion

Some of the genes under investigation, or their family members, have been associated with various types of stress conditions before. For example, the expression pattern of AtWRKY6 was related to boron deficiency, senescence and during bacterial infection (Chen et al., 2009; Kasajima et al., 2010; Robatzek and Somssich, 2002). Similarly, AtWRKY75 was reported to play a role in the regulation of phosphate deficiency and during senescence in plants (Devaiah et al., 2007; Guo et al., 2004). ROS production by NADPH oxidase, encoded by the AtRboh gene family in *Arabidopsis*, plays a crucial role in plant defense against pathogen infection (Torres and Dangl, 2005; Yun et al., 2011). Arabinogalactan proteins (AGP) participate in the growth and development of plants. Additionally, AGPs were reported to be involved in the cross-talk with salicylic acid and defense response gene PR1 (Gaspar et al., 2004). Certain indications suggest that AGPs also play a role in PCD (Chaves et al., 2002). The above studies indicate that the genes under investigation here (table 1), may have a role in the plant defense response against different stresses, including PCD. Significant change in cell death symptoms was not observed in T-DNA knockouts of these genes as well as in the knockouts in a *loh2* background. However, these genes were regulated during AT and AAL-toxin induced cell death. Based upon current studies, it can be presumed that these genes play an indirect role in relation to the PCD pathway triggered by either PQ, AT and AAL-toxin.

Slight variations in the cell death symptoms were detected in the case of single mutants for WRKY6, WRKY75, RHD2, SBT3.3, XTH21, XTH26 and AGP13 and the respective double mutants with *loh2* (supplementary tables 1-4). We were unable to observe a clear phenotype in either case. Failure to attain the phenotype, in single and double mutants, can be explained by functional redundancy. In multigene families, the mutation of a single member seldom exhibits an altered phenotype, probably due to overlapping functional redundancy. In the case of AtWRKY54 knockout lines, the resistance to pathogen infection (*Peronospora parasitica*) was not only compromised but the transient expression of several WRKY genes was also altered (Kalde et al., 2003). The *Arabidopsis*

mutants, *wrky25* and *wrky33*, were unable to develop a NaCl-sensitive phenotype independently, although increase in transcription level was observed in microarray analyses of *Arabidopsis* roots. However, *wrky25wrky33* double mutants exhibited moderate sensitivity to NaCl stress (Jiang and Deyholos, 2009). Comparable results were obtained in the case of AGP6 and AGP11 in relation to the development of viable pollen grains (Coimbra et al., 2009). Similar effects were observed in other gene families involved in plant development, hypersensitive response and senescence (McLellan et al., 2009; Motose et al., 2004). Therefore, the loss-of-function of these genes might be overcome by other members of the same gene family. Additionally, some of these target genes (SBT3.3, XTH21, XTH26 and AGP13) are directly involved in plant growth and development. Consequently, based upon the results, it can be proposed that these genes might not have a direct role in the PCD pathway when plants are challenged by different stresses triggered by PQ, AT or AAL-toxin. Moreover some of the T-DNA inserts are in the promoter region, which indicates that these genes might not be knocked out. Therefore, these mutations were unable to produce an effect during cell death induced by either PQ, AT or AAL-toxin. Wang (2008) reviewed 1084 insertion mutants in 755 *Arabidopsis* genes. He found that the T-DNA insert in the promoter results in a knockout in only 41% of the cases.

4.1. Role of zinc *Arabidopsis thaliana*11 (ZAT11) in ROS-induced programmed cell death

The *loh2/zat11-1* and *loh2/zat11-2* were the only combination of double mutants that gave significant results compared to *loh2* when leaves were treated with PQ (figure 3, supplementary table 3). The *loh2/zat11-1* and *loh2/zat11-2* double mutants exhibited much lower rates of electrolyte leakage, 4.42% and 5.52% respectively, than *loh2* (68.6%), 72 hours after treatment with PQ. Interestingly, the electrolyte leakage was also reduced in each of the double mutants when compared to their respective single mutants (12.0% and 12.7% for the single mutants, respectively). However, any altered phenotype, in relation to cell death symptoms, was not recognizable in *loh2/zat11-1* and *loh2/zat11-2* when treated with AT or

AAL-toxin. This might be due to the indirect effects of AT and AAL-toxin in comparison with PQ treatment. AT and AAL-toxin inhibit the activity of catalases and ceramide synthase, respectively. As a result, ROS levels increase inside the cell and trigger the PCD process. However, PQ induces ROS stress directly inside the chloroplasts by providing an electron to an oxygen molecule during photosynthesis. In addition, variations in cell death symptoms were not observed in the seven day old seedlings of the double mutants, in comparison to *loh2* cell death symptoms. This might be because different genes are differentially regulated at different developmental stages of the plants. PQ causes severe oxidative stress during photosynthesis, seedlings of the double mutants are unable to overcome the oxidative stress. An effect of the mode of application of PQ cannot be excluded, as PQ was taken up by the seedlings through the root system compared to spraying the leaves of the four weeks old plants.

ZAT11 is a member of the zinc finger proteins (ZFP) gene family. ZFPs play a key role in cellular functions of eukaryotes (Englbrecht et al., 2004). They are not only involved in plant growth and development but also in plant stress and activation of the defense response (Huang et al., 2009; Wang et al., 2005). Interestingly, ZAT10 plays a dual role in plant defense against salinity, heat and osmotic stress in *Arabidopsis*. ZAT10 overexpression as well as knockout and RNAi line confers tolerance to salinity and osmotic stress. ZAT10 is either functionally attributed to trigger the activation of the ROS response by activating ascorbate peroxidase2 (Apx2) and Fe-superoxide dismutase1, or it suppresses plant defense against salinity and osmotic stress (Mittler et al., 2006). ZAT12, another close relative of ZAT11, is a versatile player in plant reactions to stress conditions. ZAT12 responds to a number of abiotic stresses such as oxidative, osmotic, salinity, high light and temperature stress (Davletova et al., 2005). ZAT12 was found to be important in the regulation of cytosolic Apx1 enzymatic activity and for the expression of WRKY25 and ZAT7 during oxidative stress. The expression of ZAT7, ZAT12 and WRKY25 is increased in the *Arabidopsis* Apx1 mutant. This might be due to the accumulation of H_2O_2 in the mutant caused by the lack of Apx1 activity. ZAT7 and ZAT12 overexpressing plants were able to tolerate oxidative stress,

unlike WRKY25 transgenic plants. However, ZAT12-deficient plants were more sensitive to H_2O_2 or PQ and had reduced expression of Apx1, when compared to ZAT7 or WRKY25 mutants during oxidative stress (Rizhsky et al., 2004).

5. Conclusions

Our data suggest that ZAT11 also plays a role in PQ induced oxidative stress. Interestingly, *zat11* T-DNA mutants demonstrate increased cell death symptoms compared to the respective double mutants with *loh2* (figure 3). It might be possible that ZAT11 plays an indirect role in ROS-induced PCD. ZAT11 produced an effect, in case of *loh2/zat11* double mutants, when the mutants were subjected to oxidative stress caused by PQ. However, the specific interaction of ZAT11 and the *loh2* locus in relation to oxidative stress is unclear. Further analysis of *loh2/zat11* double mutants might be useful in order to explain the interaction of both genes. Also, incorporation of mutant plants overexpressing ZAT11 may provide a valuable clue in this regard.

The data presented in this paper demonstrates a role of ZAT11 in paraquat-induced oxidative stress. The lack of phenotype in the other types of ROS-induced oxidative stress and cell death may reflect the specificity of signaling during paraquat-induced PCD and the need of interaction with additional molecular partners. Further molecular analysis of transcriptome and metabolome in single and double mutant *zat11* backgrounds could provide valuable insights on the precise mechanisms leading to oxidative stress tolerance.

Supporting online material

http://www.rug.nl/staff/m.k.qureshi/supplementary_table_Chapter_5.pdf

Supplementary table 1: Fresh weight of wild type, single and double mutant seedlings grown on the plant growth media supplemented with different concentrations of either AT, PQ or AAL-toxin. The data was collected on the 7th day after germination in case of PQ treatment and on the 12th day in case of AT or AAL-toxin treatments. The fresh weight of ten seedlings was determined for each genotype in each condition. Data represents the mean of one seedling, ± SD.

Supplementary table 2: Chlorophyll (*chyl a* and *chyl b*) measurements for wild type, single and double mutant seedlings grown on the plant growth media supplemented with different concentrations of AT, PQ and AAL-toxin. The data was collected on the 7th day after germination in case of PQ treatment and on the 12th day in case of AT or AAL-toxin treatments. The *chyl a* and *chyl b* of ten seedlings were determined for each genotype in each condition. Data represents the mean of one seedling, ± SD.

Supplementary table 3: Cell death measurement in the leaves of wild type, single and double mutant plants treated with either PQ or AT. Four week old plants were sprayed with either 15 µM PQ or 25 mM AT. Water treated plants were used as controls. Cell death at 7, 24, 48 and 72 hours after treatments was determined as the increase in conductivity and calculated as the percent of the total conductivity obtained after boiling the samples. The data are represented as means ± SD.

Supplementary table 4: Cell death measurement in the leaves of wild type, single and double mutant plants treated with AAL-toxin. Leaves of four week old plants were infiltrated with 200 nM AAL-toxin. Water treated plants were used as controls. Cell death at 7, 24, 48 and 72 hours after treatments was determined as the increase in conductivity and calculated as the percent of the total conductivity obtained after boiling the samples. The data are represented as means ± SD.

References

1. Brandwagt BF, Mesbah LA, Takken FLW, Laurent PL, Kneppers TJA, Hille J, Nijkamp HJJ (2000) A longevity assurance gene homolog of tomato mediates resistance to *Alternaria alternata* f. sp *lycopersici* toxins and fumonisin B1. Proc Natl Acad Sci USA **97:** 4961-4966.
2. Chaves I, Regalado AP, Chen M, Ricardo CP, Showalter AM (2002) Programmed cell death induced by (β-$_{D}$-galactosyl)$_3$ Yariv reagent in *Nicotiana tabacum* BY-2 suspension-cultured cells. Physiol Plant **116:** 548-553.
3. Chen YF, Li LQ, Xu Q, Kong YH, Wang H, Wu WH (2009) The WRKY6 transcription factor modulates *PHOSPHATE1* expression in response to low Pi stress in *Arabidopsis*. Plant Cell **21:** 3554-3566.
4. Coll NS, Epple P, Dangl JL (2011) Programmed cell death in the plant immune system. Cell Death Differ **18:** 1247-1256.
5. Coimbra S, Costa M, Jones B, Mendes MA, Pereira LG (2009) Pollen grain development is compromised in *Arabidopsis agp6 agp11* null mutants. J Exp Bot **60:** 3133-3142.
6. Davletova S, Schlauch K, Coutu J, Mittler R (2005) The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in *Arabidopsis*. Plant Physiol **139:** 847-856.
7. Devaiah BN, Karthikeyan AS, Raghothama KG (2007) WRKY75 transcription factor is a modulator of phosphate acquisition and root development in *Arabidopsis*. Plant Physiol **143:** 1789-1801.

8. Englbrecht CC, Schoof H, Böhm S (2004) Conservation, diversification and expansion of C2H2 zinc finger proteins in the *Arabidopsis thaliana* genome. BMC Genomics **5:** 39.
9. Fujibe T, Saji H, Arakawa K, (2004) A methyl viologen-resistant mutant of *Arabidopsis*, which is allelic to ozone-sensitive *rcd*1, is tolerant to supplemental ultraviolet-B irradiation. Plant Physiol **134:** 275-285.
10. Gaspar YM, Nam J, Schultz CJ, Lee LY, Gilson PR, Gelvin SB, Bacic A (2004) Characterization of the *Arabidopsis* lysine-rich arabinogalactan protein AtAGP17 mutant (*rat1*) that results in a decreased efficiency of *Agrobacterium transformation*. Plant Physiol **135:** 2162-2171.
11. Gadjev I, Vanderauwera S, Gechev TS, Laloi C, Minkov IN, Mittler R, Breusegem FV, Shulaev V, Apel K, Inze D (2006) Transcriptomic footprints disclose specificity of reactive oxygen species signaling in *Arabidopsis*. Plant Physiol **141:** 436-445.
12. Gechev T, Gadjev I, Van Breusegem F, Inze D, Dukiandjiev S, Toneva V, Minkov I (2002) Hydrogen peroxide protects tobacco from oxidative stress by inducing a set of antioxidant enzymes. Cell Mol Life Sci **59:** 708-714.
13. Gechev TS, Gadjev IZ, Hille J (2004) An extensive microarray analysis of AAL-toxin-induced cell death in *Arabidopsis thaliana* brings new insights into the complexity of programmed cell death in plants. Cell Mol Life Sci **61:** 1185-1197.
14. Gechev TS, Hille J (2005) Hydrogen peroxide as a signal controlling plant programmed cell death. J Cell Biol **168:** 17-20.
15. Gechev TS, Van Breusegem F, Stone JM, Denev I, Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. BioEssays **28:** 1091-1101.
16. Gechev TS, Ferwerda MA, Mehterov N, Laloi C, Qureshi MK, Hille J (2008) *Arabidopsis* AAL-toxin-resistant mutant *atr1* shows enhanced tolerance to programmed cell death induced by reactive oxygen species. Biochem Biophys Res Commun **375:** 639-644.
17. Gunawardena AH (2008) Programmed cell death and tissue remodelling in plants. J Exp Bot **59:** 445-51.
18. Guo Y, Cai Z, Gan S (2004) Transcriptome of *Arabidopsis* leaf senescence. Plant Cell Environ **27:** 521-549.
19. Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol **141:** 312-322.
20. Huang XY, Chao DY, Gao JP, Zhu MZ, Shi M, Lin HX (2009) A previously unknown zinc finger protein, DST, regulates drought and salt tolerance in rice via stomatal aperture control. Genes Dev **23:** 1805-1817.

21. Inskeep WP, Bloom PR (1985) Extinction coefficients of chlorophyll *a* and *b* in n,n-dimethylformamide and 80% acetone. Plant Physiol **77:** 483-485.
22. Ito J, Fukuda H (2002) ZEN1 is a key enzyme in the degradation of nuclear DNA during programmed cell death of tracheary elements. Plant Cell **14:** 3201-3211.
23. Jiang Y, Deyholos MK (2009) Functional characterization of *Arabidopsis* NaCl-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. Plant Mol Biol **69:** 91-105.
24. Kalde M, Barth M, Somssich IE, Lippok B (2003) Members of the *Arabidopsis* WRKY group III transcription factors are part of different plant defense signaling pathways. Mol Plant Microbe Interact **16:** 295-305.
25. Kasajima I, Ide Y, Yokota Hirai M, Fujiwara T (2010) WRKY6 is involved in the response to boron deficiency in *Arabidopsis thaliana*. Physiol Plant **139:** 80-92.
26. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods **25:** 402-408.
27. McLellan H, Gilroy EM, Yun BW, Birch PR, Loake GJ (2009) Functional redundancy in the *Arabidopsis* Cathepsin B gene family contributes to basal defence, the hypersensitive response and senescence. New Phytol **183:** 408-418.
28. Mittler R, Kim Y, Song L, Coutu J, Coutu A, Ciftci-Yilmaz S, Lee H, Stevenson B, Zhu JK (2006) Gain- and loss-of-function mutations in Zat10 enhance the tolerance of plants to abiotic stress. FEBS Lett **580:** 6537-6542.
29. Motose H, Sugiyama M, Fukuda H (2004) A proteoglycan mediates inductive interaction during plant vascular development. Nature **429:** 873-878.
30. Qureshi MK, Radeva V, Genkov T, Minkov I, Hille J, Gechev TS (2011) Isolation and characterization of *Arabidopsis* mutants with enhanced tolerance to oxidative stress. Acta Physiol Plant **33:** 375-382.
31. Rizhsky L, Davletova S, Liang H, Mittler R (2004) The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. J Biol Chem **279:** 11736-11743.
32. Robatzek S, Somssich IE (2002) Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes Dev **16:** 1139-1149.
33. Spassieva SD, Markham JE, Hille J (2002) The plant disease resistance gene Asc-1 prevents disruption of sphingolipid metabolism during AAL-toxin-induced programmed cell death. Plant J **32:** 561-572.
34. Torres MA, Dangl JL (2005) Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr Opin Plant Biol **8:** 397-403.
35. Van Breusegem F, Dat J (2006) Reactive oxygen species in plant cell death. Plant Physiol **141:** 384-390.

36. Wang L, Pei Z, Tian Y, He C (2005) OsLSD1, a rice zinc finger protein, regulates programmed cell death and callus differentiation. Mol Plant Microbe Interact **18:** 375-384.
37. Wang YH (2008) How effective is T-DNA insertional mutagenesis in *Arabidopsis* ? J Biochem Tech **1:** 11-20.
38. Willekens H, Chamnongpol S, Davey M, Schraudner M, Langebartels C, Van Montagu M, Inzé D, Van Camp W (1997) Catalase is a sink for H_2O_2 and is indispensable for stress defence in C-3 plants. EMBO J **16:** 4806-4816.
39. Yun BW, Feechan A, Yin M, Saidi NB, Le Bihan T, Yu M, Moore JW, Kang JG, Kwon E, Spoel SH, Pallas JA, Loake GJ (2011) S-nitrosylation of NADPH oxidase regulates cell death in plant immunity. Nature **478:** 264-268.
40. Zimmermann P, Heinlein C, Orendi G, Zentgraf U (2006) Senescence-specific regulation of catalases in *Arabidopsis thaliana* (L.) Heynh. Plant Cell Environ **29:** 1049-1060.

Summary

Summary

Plants are always at risk of fungal pathogen infections. These fungal pathogens can be divided into biotrophs and necrotrophs. Biotrophic fungal pathogens feed on living plant tissues using specialized feeding structures, whereas necrotrophic fungi use dead plant tissue as a nutrient source. The latter often secrete toxins that trigger cell death at the site of infection. The necrotrophic fungus *Alternaria alternata* f. sp. *lycopersici* triggers cell death by secreting host-selective phytotoxins. These AAL-toxins are well characterized inducers of programmed cell death (PCD). AAL-toxins inhibit the enzyme ceramide synthase and this disruption of sphingolipid metabolism triggers PCD. AAL-toxins also trigger the accumulation of reactive oxygen species (ROS) prior to the PCD process.

ROS are obligate byproducts of photosynthesis and respiration. Biotic stresses, as mentioned above and abiotic stresses such as salinity, drought, ozone and herbicides like 3-aminotriazole (AT) and paraquat (PQ) can trigger oxidative stress and subsequently ROS-induced PCD. Excessive amounts of ROS can cause damage to proteins, pigments, lipids and DNA resulting in cell destruction. ROS can induce PCD or protective mechanisms in a dose dependent manner.

PCD is considered as a genetically controlled process in the life cycle of eukaryotes. It is essential for plant growth and development and during interactions with the environment. In some cases like pathogen attack and abiotic stresses, PCD is threatening for the plant survival. Pathogen attack may trigger a hypersensitive response (HR) at the site of infection. The HR is a plant defense response, which restricts pathogen spread by initiating cell death in and around the site of infection.

The research in this thesis is based upon the role of AAL-toxins and ROS in the regulation of PCD in the model plant species, *Arabidopsis thaliana*. AAL-toxins disrupt sphingolipid metabolism resulting in PCD, as illustrated in chapter 1. AAL-toxins are analogous to sphinganine and disrupt the activity of one of the ceramide synthases. Ceramide synthases are involved in sphingolipid metabolism and as such in the synthesis of complex sphingolipids from sphinganine. Sphingolipids are important constituents of the endomembrane system in eukaryotes. Different kinds of ROS and their role in the PCD signaling cascade are discussed in this chapter.

ROS chemistry together with the intensity of signal perception, site of ROS production, age and history of the plant, and interaction with other signaling molecules are important for the ROS driven PCD signaling pathway. The activity of different players, like mitogen activated protein kinases, calcium influx, an array of transcription factors and various proteases, results in the final outcome.

In chapters 2, 3 and 4, a forward genetics approach is utilized for the identification of genes, which might have a regulatory role in AAL-toxins- and ROS-induced PCD. In chapter 5 the role of 8 genes in PCD is studied utilizing a reverse genetics approach.

Different kinds of ROS have the ability to initiate PCD. In chapter 2, the role of hydrogen peroxide (H_2O_2) in PCD is studied. The exogenous application of AT inhibits the activity of the ROS scavenging enzyme, catalase. This results in the endogenous accumulation of H_2O_2 triggering cell death. In this chapter transcriptomes are evaluated of AAL-toxins sensitive and one of the resistant mutants during cell death induced by H_2O_2. The sensitive and tolerant mutants were designated as *loh2* (LAG one homologue2) and *atr1* (AAL-toxin resistant), respectively. The *atr1* mutant was identified through ethane methyl sulfonate (EMS) induced mutation in *loh2* and subsequent screening of progeny seeds for AAL-toxins resistance. The chapter discusses two potential distinct pathways activated by AT-induced oxidative stress in *loh2* and *atr1*. One pathway, which is active in *loh2* and *atr1*, is responsible for the growth inhibition. Another pathway, active only in *loh2*, is responsible for cell death.

In chapter 3, the characterization of an additional eight *atr* mutants (*atr2*-*atr9*) with enhanced tolerance to AAL-toxins is described. The *loh2* mutant plants exhibited classical PCD symptoms when subjected to AAL-toxins, whereas *atr* mutants developed less cell death symptoms. The mutants were characterized with respect to oxidative stress by growing them on media supplemented either with AT or PQ. Some of the mutants showed enhanced tolerance to ROS and remained viable compared to *loh2*, which developed necrotic lesions. Additionally, six of the mutants show a dwarf phenotype with altered leaf shape when grown in stress free conditions. The *atr2* mutant exhibited delayed flowering and senescence. This

indicates that some of the mutant gene(s) associated with tolerance to oxidative stress in *atr* mutants may also be involved in regulating plant growth and development.

The potential role of the genes mutated in the *atr* mutants with respect to PCD induced by AAL-toxins, PQ and AT presented a need to isolate these genes and further characterize these mutants. The identification of two genes of the *atr* mutants (*atr2* and *atr7*) through a map-based cloning approach and subsequent sequencing is carried out in chapter 4. These two mutant phenotypes segregate as monogenic traits in genetic analysis. Mapping narrowed down the area around the two mutated genes to about 100 kb genomic regions on chromosome five. As further mapping was difficult due to lack of additional recombinants a massive parallel sequencing strategy was utilized. Subsequently, we identified the *atr2* and *atr7* mutations in two novel genes At5g19210 and At5g21280, respectively. The analysis of DNA and protein sequences indicated that *atr2* is homologous to DEAD-box RNA helicase proteins, whereas *atr7* encodes a protein with similarities to a hydroxyproline-rich glycoprotein.

The role of the Zinc *Arabidopsis thaliana*11 (ZAT11) gene in the PCD signaling pathway has been shown in chapter 5. ZAT11 is a member of the zinc finger protein family. We selected eight genes, regulated during AAL-toxins or AT induced cell death in *loh2*, with the possibility of playing a role in regulating PCD. The selection of regulated genes was made at an early time-point before any visible signs of cell death. Mutants of seven genes and also when crossed into the *loh2* background showed no specific phenotypes under different stress conditions. However, two independent *loh2/zat11* mutants exhibited tolerance to oxidative stress induced by PQ. The same concentration of PQ produces lethal effects in *loh2* plants. The chapter provides indications that ZAT11 plays a potential role in the PCD signaling pathway induced by ROS through an unknown mechanism.

In conclusion, the identification of regulators of PCD is important for the understanding of this complex pathway. A better understanding of PCD can help us to develop more effective breeding strategies against biotic and abiotic stress factors. In this thesis a potential link is provided between AAL-toxins and ROS during the

PCD process. Through molecular and functional genomics approaches the role of novel genes in PCD has been established.

Samenvatting

Samenvatting

Schimmelinfecties vormen een belangrijk risico voor planten. De schimmel pathogenen kunnen worden onderverdeeld in biotrofe en necrotrofe schimmels. Biotrofe schimmels voeden zich met levend plantenweefsel met behulp van gespecialiseerde voeding structuren, terwijl necrotrofe schimmels dood plantenweefsel gebruiken als voedingsbron. Deze laatste groep scheidt vaak giftige stoffen uit die celdood veroorzaken op de plaats van infectie. De necrotrofe schimmel *Alternaria alternata* f. sp. *lycopersici* veroorzaakt celdood door het afscheiden van gastheer-selectieve fytotoxinen. Deze AAL-toxines zijn goed gekarakteriseerde induceerders van geprogrammeerde celdood (PCD). AAL-toxines remmen het enzym *ceramide synthase* en daarmee het sfingolipiden metabolisme wat leidt tot PCD. Voorafgaand aan het proces van PCD leidt blootstelling aan AAL-toxines tot ophoping van reactieve vormen van zuurstof (reactive oxygen species; ROS).

ROS zijn obligate bijproducten van de fotosynthese en de ademhalingsketen. Biotische stress, zoals hierboven vermeld en abiotische stress, zoals verzilting, droogte, ozon en herbiciden (zoals 3-aminotriazole (AT) en paraquat (PQ)) kunnen oxidatieve stress veroorzaken. Overmatige hoeveelheden ROS kunnen schade veroorzaken aan eiwitten, pigmenten, lipiden en DNA resulterend in celdood. ROS kunnen PCD veroorzaken maar ook, op een dosis-afhankelijke manier, beschermende mechanismen induceren.

PCD is een genetisch gecontroleerd proces dat deel uitmaakt van de levenscyclus van eukaryote cellen. Het is een essentieel proces voor zowel groei als ontwikkeling van planten maar ook essentieel bij interacties van de plant met zijn omgeving. PCD kan in sommige gevallen echter ook een bedreiging vormen voor overleving van de plant, zoals aanvallen door ziekteverwekkers en abiotische stress. Aanval door een pathogeen kan leiden tot een overgevoeligheidsreactie (Hyper sensitive Responce; HR) op de plaats van infectie. De HR reactie is een verdediging van planten, die pathogeenverspreiding beperkt door het initiëren van celdood in en rond de plaats van de infectie.

Het onderzoek in dit proefschrift is gebaseerd op de rol van AAL-toxines en ROS in de regulatie van PCD in de modelplant, *Arabidopsis thaliana*. AAL-toxine verstoort sfingolipide metabolisme resulterend in PCD, zoals geïllustreerd in hoofdstuk 1. AAL-toxinen zijn analogen van sfinganine en verstoren de activiteit van een van de ceramide synthases. Ceramide synthases zijn betrokken bij sfingolipiden metabolisme en als zodanig betrokken bij de synthese van complexe sfingolipiden uit sfinganine. Sfingolipiden zijn belangrijke bestanddelen van het endomembraan systeem in eukaryoten. Verschillende soorten ROS en hun rol in de PCD-signaleringscascade worden besproken in dit hoofdstuk. ROS chemie samen met de intensiteit van de signaal waarneming, plaats van de ROS productie, leeftijd en geschiedenis van de plant, en de interactie met andere signaleringsmoleculen zijn belangrijk voor de ROS gedreven PCD signaal route. De activiteit van verschillende factoren zoals mitogeen geactiveerde eiwit kinases, calcium influx, een cascade aan transcriptiefactoren en diverse proteases, bepalen het uiteindelijk resultaat.

In de hoofdstukken 2, 3 en 4, wordt een genetische rechttoe rechtaan benadering gebruikt voor de identificatie van genen met een mogelijke regulerende rol in AAL-toxine- en ROS-geïnduceerde PCD. In hoofdstuk 5 wordt de rol van 8 genen in PCD bestudeerd met behulp van een “reverse genetics” aanpak.

Verschillende ROS hebben de mogelijkheid om PCD te initiëren. In hoofdstuk 2 wordt de rol van waterstofperoxide (H_2O_2) in PCD bestudeerd. De exogene toepassing van AT remt de activiteit van het ROS opruimende enzym *catalase*. Dit resulteert in endogene accumulatie van H_2O_2 wat vervolgens celdood initieert. In dit hoofdstuk worden de transcriptomen geëvalueerd van AAL-toxine gevoelige en één van de AAL-toxine resistente mutanten gedurende H_2O_2 geïnduceerde celdood. De gevoelige en tolerante mutanten werden respectievelijk aangeduid als *loh2* (LAG one homologue2) en *atr1* (AAL-toxine resistent). De *atr1* mutant werd geïdentificeerd door middel van een ethaan methyl sulfonaat (EMS) geïnduceerde mutatie in *loh2* en de daarop volgende screening van de nakomelingen op AAL-toxine resistentie. In het hoofdstuk worden twee mogelijke, en potentieel verschillende, routes besproken die door AT-geïnduceerde oxidatieve stress in *loh2* en *atr1* worden geactiveerd. Eén route, die actief is in *loh2* en *atr1*, is

verantwoordelijk voor groeiremming. Een andere route, alleen actief in *loh2*, is verantwoordelijk voor celdood.

In hoofdstuk 3 wordt de karakterisering van nog eens acht *atr* mutanten (*atr2*-*atr9*) met verhoogde weerstand tegen AAL-toxines beschreven. De *loh2* mutant planten vertonen klassieke PCD symptomen na blootstelling aan AAL-toxine, terwijl *atr* mutanten veel minder celdood symptomen vertonen. Karakterisering van de mutanten, met betrekking tot oxidatieve stress, vond plaats door ze te groeien op media waar AT of PQ aan was toegevoegd. Sommige mutanten vertoonden een verhoogde tolerantie voor ROS en bleven levensvatbaar in vergelijking tot *loh2* die necrotische lesies vertoonde. Bovendien, vertoonden zes van de mutanten, wanneer ze groeien onder stress-vrije omstandigheden, een dwerg fenotype met een veranderde bladvorm. De *atr2* mutanten vertonen een vertraagde bloei en een vertraagd afsterven. Dit geeft aan dat sommige van de gemuteerde gen(en) die geassocieerd zijn met tolerantie voor oxidatieve stress in *atr* mutanten ook betrokken zouden kunnen zijn bij de regulatie van groei en ontwikkeling van planten.

De mogelijke rol van de genen die in de *atr* mutanten zijn gemuteerd en hierdoor resistentie geven tegen AAL-toxines, PQ en AT geeft de noodzaak weer om deze (gemuteerde) genen te isoleren en verder te karakteriseren. De identificatie van twee genen van de *atr*-mutanten (*atr2* en *atr7*) door middel van "map-based cloning" gevolgd door sequentie analyse wordt beschreven in hoofdstuk 4. Deze twee mutante fenotypes segregeren in een genetische analyse als monogene eigenschappen. Door middel van kartering konden de gebieden rond de twee gemuteerde genen teruggebracht worden tot gebieden van ongeveer 100 kb gelegen op chromosoom vijf. Omdat verdere kartering moeilijk was, werd er gebruik gemaakt van een nieuwe sequentie analyse techniek ('massive parallel sequencing') waarmee de DNA volgordes van de 100 kb gebieden konden worden opgehelderd. Vervolgens werden de *art2* en *atr7* mutaties geïdentificeerd in twee nieuwe genen respectievelijk At5g19210 en At5g21280. De analyse van DNA en eiwitsequenties gaven aan dat *atr2* homoloog is met DEAD-box RNA-helicase eiwitten, terwijl *atr7*

codeert voor een eiwit dat overeenkomsten vertoont met een hydroxyproline-rijk glycoproteine.

De rol van het Zinc *Arabidopsis thaliana*11 (ZAT11) gen in de PCD-signaleringsroute is aangetoond in hoofdstuk 5. ZAT11 maakt deel uit van de zink-vinger eiwit familie. We hebben acht genen geselecteerd die mogelijk een rol spelen in de regulatie van PCD en die gereguleerd worden door AAL-toxine of AT geïnduceerde celdood in *loh2*. De selectie van gereguleerde genen werd gedaan in een vroeg stadium van het PCD proces, voordat er enig teken van celdood zichtbaar was. Mutanten van zeven genen (ook in de *loh2* achtergrond) vertoonden geen specifieke fenotypes onder verschillende stress condities. Echter, twee onafhankelijke *loh2/zat11* mutanten vertoonden tolerantie voor oxidatieve stress veroorzaakt door PQ. Dezelfde concentratie PQ produceert letale effecten in *loh2* planten. Hoofdstuk 5 geeft aanwijzingen dat ZAT11 een mogelijke rol in de PCD-signalerings route speelt die door ROS, via een onbekend mechanisme, wordt geïnduceerd.

Kortom, de identificatie van regulatoren van PCD is belangrijk voor het begrijpen van dit complexe proces. Een beter begrip van PCD kan ons helpen bij het ontwikkelen van effectievere veredelingsstrategieën tegen biotische en abiotische stress factoren. In dit proefschrift wordt een potentieel verband gelegd tussen AAL-toxines en ROS in het proces van PCD. Middels een moleculair genetische en functioneel genomische aanpak is de rol van nieuwe genen in PCD aangetoond.

Acknowledgements

Acknowledgements

In the name of Allah (God), the most merciful and the most beneficent. I offer my humblest thanks to the Holy Prophet Muhammad (Peace be upon him), a torch of guidance and knowledge for me.

Though words may never say what we want to express, still they are most appropriate for expressing our emotions. The book you are looking at, is the abstract of many desperate attempts. Although some of those attempts were unable to bear fruit. During those attempts, I was guided, helped, motivated, encouraged and supported by many people whom I feel to acknowledge.

First of all, I wish to record my sincerest gratitude to my promotor Prof. dr. J. Hille for accepting me as a PhD student even though “I was married”. I am thankful for his guidance and for conveying a spirit of adventure with regard to research. I greatly appreciate his “sugar quoted” criticism, which sometimes turns into “sugar free” when it comes to meet a deadline. But, he never lost his patience and provided me with motivation when I needed it the most. With his valuable comments, I was able to finalize the writing process for my thesis. I would also like to thank my co-promotor Dr. T.S. Gechev for helping me in experimenting with different ideas, which resulted in nice publications. I am also grateful for his critical opinion on my manuscript. The preparation of this thesis would not have been possible without his valuable contribution. Special thanks to Prof. dr. J.T.M. Elzenga, Prof. dr. J.D. van Elsas and Prof. dr. J. Kok for being members of the reading committee. I greatly appreciate their remarks on my thesis.

I would like to appreciate the company of my colleagues. I am thankful to dr. S. Neerakkal for providing guidance and a helping hand during some of the critical experiments. Big thanks to I. Rasul for technical support and for keeping my plants alive in my absence. I will never forget the phrase of my dear colleague Eelco (dr. T.E. Wallaart), “I am always busy but I can make time for you”. Apart from many other favours that he provided me, I am thankful for making the samenvatting (Dutch translation) for my thesis summary. I would like to acknowledge dr. J. Warmink for creating a pleasurable atmosphere in the lab. I am also thankful to him for becoming one of my paranymphs. Thanks to my former colleagues Aukje, Bert,

dr. Jos, Marcel, Margriet, dr. Oktavia and dr. Reza for providing me with their expertise in the early stages of my studies. I would like to pay my gratitude to Prof. dr. J.T.M. Elzenga for letting me use the facilities of the department of Plant Physiology during the last year of my PhD. I am also thankful to G. Telkamp and M. Staal for their technical support.

I am grateful to the Higher Education Commission, Pakistan for providing me with the financial support of my PhD. I also appreciate Nuffic, The Netherlands organization for international cooperation in higher education, for official work processing for Pakistani PhD students.

I enjoyed the company of the wonderful Pakistani community during my stay in Groningen. I would like to appreciate dr. Adnan, Afzal, A. Somro, Aisha, dr. Arslan, Atta, dr. Ikram, Irfan, Ishtiaq, Javed, Latif, Mansoor, Nadia, Nazia, Tanveer, dr. Tariq, Samin, Sulman, Umar and dr. Waqas for providing me with their company at many different junctures of my stay in Groningen. Special thanks to Ali, Bilal, Ehsan, dr. Imran, dr. Munir, dr. Shahbaz, Rashid, dr. Younas and Zia for helping me during a variety of occasions. I enjoyed our weekly dinners where some of you exhibited fantastic cooking skills and deliberate and creative jokes.

I do not have words at my command to express my gratitude and profound admiration to my affectionate parents. They provided me with their continuous support and love during my entire life. I greatly appreciate how they boosted my morale on a daily basis from thousands of miles away. I wish them health and a long life. I would also like to appreciate my family-in-law for their support. I cannot forget my father-in-law (late) and my brother-in-law K.A. Qureshi (late) who helped me in arranging all the necessary documents so that my family could stay in The Netherlands. Unfortunately, they are not here in this world to share my happiness, but I am sure that they are excited for me somewhere in heaven.

Lastly, I would like to express my gratitude towards my wife, who supported me during my PhD. Apart from her support during the entire PhD period, she relieved me from almost all of my family obligations during the last year of my PhD so that I could concentrate on the writing of my thesis. My children A. Kamran and M.R. Kamran always provided me with strength and a sense of responsibility during

my studies. When I used to come home after a long hard day's work and both of them force me to become a horse. It used to be the most relaxing message that I could have. I also want to apologize for sometimes returning home very late and not spending time with them. The only thing I can say is that I will try to become a good father.

The best possible words I can say to all of them in return are Jazak-a-Allah (Allah will reward you).

Muhammad Kamran Qureshi

March, 2012

Groningen

Curriculum Vitae

Muhammad Kamran Qureshi was born on September 27, 1981 in Multan, Pakistan. He finished his basic education in the year 2000 from Nishat High School (Pvt.), Multan and from the Government College, Bosan Road, Multan. In 2004, he received the Bachelor of Science specializing in Plant Breeding & Genetics from the University of Agriculture (UAF), Faisalabad, Pakistan. He received his Master of Science in 2006 from UAF, Faisalabad. The main topic of his M.Sc. thesis was "Effect of drought tolerant alleles on agronomic traits using DNA polymorphic genotypes in upland cotton". In 2006, he successfully undertook a nationwide Graduate Record Examination test, which resulted in his selection for the overseas PhD scholarships announced by the Higher Education Commission (HEC), Pakistan. He started his research in the Department Molecular Biology of Plants, University of Groningen, The Netherlands in October 2007. He has been permitted to defend his thesis entitled "Characterization of genes involved in the regulation of programmed cell death in *Arabidopsis thaliana*" in May 2012.

Courses taken

1. Molecular Biology of Fungi and Plants
2. Radiation Protection Course (Level 5B)
3. GBB introduction course
4. Dynamic Presentation Course
5. Dutch Language Course (Level 1)

Conferences and presentations

1. ALW Discussie Platform Experimentele Planten Wetenschappen (EPW) (7 and 8 April 2008, Lunteren, The Netherlands)
2. ALW Discussie Platform Experimentele Planten Wetenschappen (EPW) (19 and 20 April 2010, Lunteren, The Netherlands)
3. EMBN Workshop on Membrane Proteins and Proteomics (27th to 31st January, 2008, University of Groningen, The Netherlands)

4. Poster at GBB symposium, September 12, 2008, University of Groningen, The Netherlands, Title: Functional characterization of genes involved in the regulation of programmed cell death in *Arabidopsis thaliana*
5. Poster at GBB symposium, September 11, 2009, University of Groningen, The Netherlands, Title: Characterization of Phoenix mutants in *Arabidopsis thaliana*
6. Poster at GBB symposium, September 09, 2011, University of Groningen, The Netherlands, Title: Characterization of genes involved in the regulation of Programmed Cell Death in *Arabidopsis thaliana*
7. Botanical excursion, November 11, 2011, Delfzijl, The Netherlands

Publications

1. Gechev TS, Ferwerda MA, Mehterov N, Laloi C, **Qureshi MK**, Hille J (2008) Identification of genes involved in the regulation of programmed cell death in *Arabidopsis thaliana*. Biochem Biophys Res Commun **375:** 539-544.
2. Gechev T, Ferwerda M, Mehterov N, Radeva V, Denev I, **Qureshi K**, Laloi C, Toneva V, Minkov I, Hille J (2009) Mutational analysis to dissect oxidative and abiotic stress in *Arabidopsis thaliana.* In: Q.Y. Shu (ed.) Induced Plant Mutations in the Genomics Era **pp:** 147-150.
3. **Qureshi MK**, Radeva V, Genkov T, Minkov I, Hille J, Gechev TS (2011) Isolation and characterization of *Arabidopsis* mutants with enhanced tolerance to oxidative stress. Acta Physiol Plant **33:** 375-382.
4. **Qureshi MK**, Sujeeth N, Gechev TS, Hille J (2012) The zinc finger protein ZAT11 modulates paraquat-induced programmed cell death in *Arabidopsis thaliana* (Submitted to Int J Mol Sci)